

# PCR Applications Manual

3<sup>rd</sup> edition



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<b>1</b>	<b>Introduction</b>	<b>Page</b>
1.1	Principles of PCR and RT-PCR .....	9
1.2	The Evolution of PCR .....	11
1.3	Purpose of this PCR Applications Manual.....	15
<b>2</b>	<b>General Guidelines</b>	<b>Page</b>
2.1	Preventing Contamination in the PCR Laboratory.....	19
2.1.1	Setting Up the PCR Laboratory Space Correctly.....	20
2.1.2	Cultivating Laboratory Habits That Prevent Cross-Contamination .....	21
2.1.3	Using Uracil N-Glycosylase To Eliminate Carryover Contamination .....	26
2.2	Factors To Consider When Setting Up a PCR Laboratory .....	27
2.2.1	Equipment Required.....	27
2.2.2	Choosing the Correct Enzymes for PCR and RT-PCR .....	29
2.2.3	Other Important Reaction Components .....	33
2.2.4	How Cycling Parameters Affect a PCR .....	36
2.3	Typical Workflow for PCR/RT-PCR .....	37
<b>3</b>	<b>Primer Design and Template Preparation</b>	<b>Page</b>
3.1	Primer Design .....	41
3.1.1	Strategies for Designing Good Primers .....	41
3.1.2	Design of PCR Primers: Helpful Web Sites that Contain Tips, Software, and Sequences.....	42
3.1.3	Using the Primers in PCRs.....	43
3.2	Template Preparation .....	45
3.2.1	Overview .....	45
3.2.2	Products for Manual and Automated Isolation of Nucleic Acids.....	48
3.3	Protocols for Isolation of Typical Templates .....	54
3.3.1	Isolation of High Molecular Weight Nucleic Acids from Cultured Cells with the HIGH PURE PCR Template Preparation Kit.....	54
3.3.2	Isolation of Total RNA from Whole Blood with the HIGH PURE RNA Isolation Kit .....	57
3.3.3	Isolation of cDNA with the HIGH PURE PCR Product Purification Kit.....	60

4	General PCR Methods	Page
	PCR Protocol Selection Guide.....	65
4.1	Basic PCR.....	66
4.1.1	Hot Start PCR - The new Standard.....	66
4.1.2	Conventional PCR.....	74
4.2	High Fidelity PCR.....	79
4.2.1	Reagents and Equipment Required.....	79
4.2.2	General Considerations for High Fidelity PCR.....	80
4.2.3	Protocols for High Fidelity PCR.....	81
4.2.4	Typical Results.....	90
4.3	Long Template PCR.....	92
4.3.1	Reagents and Equipment Required.....	92
4.3.2	General Considerations for Long Template PCR.....	93
4.3.3	Protocols for Long Template PCR.....	94
4.4	Amplification of Difficult Templates.....	101
4.4.1	Reagents and Equipment Required.....	101
4.4.2	General Considerations for Amplification of Difficult Templates.....	102
4.4.3	Protocols for Amplification of Difficult Templates (for Targets up to 5 kb).....	103
4.4.4	Typical Results.....	107
4.5	Guidelines for Optimizing PCR.....	108
4.5.1	Reagents and Equipment Required.....	108
4.5.2	General Considerations for Preventing Carryover.....	109
4.5.3	Protocols for Preventing Carryover.....	110
4.6	Preventing Carryover.....	117
4.6.1	Choose the Appropriate Enzyme.....	117
4.6.2	Use Highly Purified Templates and Primers.....	117
4.6.3	Design Primers Carefully.....	117
4.6.4	Use the Highest Quality Nucleotides.....	118
4.6.5	Minimize Pipetting Steps with Convenient Master Reagent Mixes.....	119
4.6.6	Optimize the Reaction Components.....	120
4.6.7	Optimize Reaction Temperatures and Times.....	123

<b>5</b>	<b>Basic RT-PCR Methods</b>	<b>Page</b>
5.1	Factors to Consider in RT-PCR.....	127
5.1.1	Choosing a One-Step or a Two-Step Procedure .....	128
5.1.2	Choosing the RT-PCR Enzymes .....	128
5.1.3	Choosing the Primers for Reverse Transcription .....	130
5.1.4	Preparation and Handling of Template RNA.....	133
5.2	Using Protector RNase Inhibitor.....	134
5.2.1	How To Use Protector RNase Inhibitor .....	134
5.3	One-Step RT-PCR.....	135
5.3.1	Reagents and Equipment Required .....	135
5.3.2	General Considerations for One-Step RT-PCR.....	136
5.3.3	Protocols for One-Step RT-PCR.....	137
5.4	Two-Step RT-PCR .....	145
5.4.1	Reagents and Equipment Required .....	145
5.4.2	General Considerations for Two-Step RT-PCR.....	146
5.4.3	Protocols for the Reverse Transcription Step (Templates up to 14 kb).....	147
5.4.4	Protocols for the PCR Step .....	154
5.4.5	Typical Results of Two-Step RT-PCR.....	155
<b>6</b>	<b>Post-PCR Purification and Cloning</b>	<b>Page</b>
6.1	Purification of PCR Products.....	159
6.1.1	Purification of PCR Products with the HIGH PURE PCR Product Purification Kit.....	160
6.1.2	HIGH PURE PCR Product Purification Kit: Changed Protocol for Purification of Large DNA Fragments (4.5 to >30 kb).....	163
6.1.3	High Throughput Purification of PCR Products with the HIGH PURE 96 UF Cleanup System .....	167
6.1.4	Elution of PCR Products from Agarose Gel Slices with the Agarose Gel DNA Extraction Kit.....	172
6.2	Cloning of PCR Products.....	175
6.2.1	Overview: Cloning of PCR Products .....	175
6.2.2	Some Useful Kits for Cloning PCR Products.....	178
6.2.3	Procedure for Cloning up to 10 kb PCR Products with the PCR Cloning Kit (Blunt End).....	183
6.2.4	Procedure for Cloning Long (7– 36 kb) PCR Products with the Expand Cloning Kit .....	184
6.2.5	Activity of Restriction Enzymes in Standard Taq DNA Polymerase Buffer .....	189
6.2.6	Activity of Restriction Enzymes in Pwo SuperYield DNA Polymerase Buffer.....	190

<b>7</b>	<b>Real-Time PCR Methods</b>	<b>Page</b>
7.1	Introduction .....	195
7.2	Real-Time PCR Assay Formats .....	198
7.3	Quantification Methods for Real-Time PCR .....	203
7.4	Product Characterization and Genotyping by Melting Curve Analysis .....	207
7.5	Real-Time PCR Instruments Available from Roche Applied Science.....	211
7.6	Real-Time PCR Reagents.....	215
7.7	Published Examples of Applications for the LightCycler® Carousel-Based Systems.....	224
<b>8</b>	<b>Applications</b>	<b>Page</b>
	General Introduction .....	229
8.1	Multiplex PCR Using the FastStart High Fidelity PCR System.....	230
8.2	Specific Amplification of Difficult PCR Products from Small Amounts of DNA Using FastStart Taq DNA Polymerase.....	238
8.3	FastStart Taq DNA Polymerase Is Ideally Suited for RT-PCR of Laser Captured Microdissected Material .....	242
8.4	Cloning of mRNAs and Rapid Screening by Direct Colony PCR with the FastStart PCR Master .....	244
8.5	FastStart High Fidelity PCR System Simplifies Study of Epigenetics and DNA Methylation .....	250
8.6	Analysis of DNA Methylation Patterns at the BRCA1 CpG Island.....	254
8.7	Comparison of Several Hot-Start Taq DNA Polymerases for Detection of Differentially Expressed Genes by GeneFishing .....	259
8.8	Transcriptional Organization of the O Antigen Biosynthesis Cluster in the GC-Rich Bacterium Burkholderia cenocepacia .....	262
8.9	Transcriptional Analysis of a Retroviral Vector System that Transfers Intron-Containing Genes .....	265
8.10	Quantification of BRCA1 Expression Levels with Standard Roche RT-PCR Reagents: A Sensitive Method for Detecting Low Amounts of Transcripts.....	271
8.11	Tailor-made Solutions Exemplified with the High-throughput 5' RACE Kit .....	276
<b>9</b>	<b>Appendix</b>	<b>Page</b>
A	Troubleshooting.....	285
B	General Information.....	293
C	Ordering Information.....	299
D	Abbreviations .....	315
E	References .....	318
F	Index.....	325
G	Trademarks and License Disclaimers.....	334

*Chapter 1*  
**Introduction**



**1**



<b>1</b>	<b>Introduction</b>	<b>Page</b>
1.1	Principles of PCR and RT-PCR.....	9
1.2	The Evolution of PCR .....	11
1.3	Purpose of this PCR Applications Manual.....	15

# 1. Introduction

## 1.1 Principles of PCR and RT-PCR

If DNA is the master blueprint of life, DNA polymerase is the molecule most responsible for replication and dissemination of the blueprint. Without DNA polymerase, most living organisms could not generate future progeny or evolve.

The principal function of DNA polymerase is to synthesize new strands of DNA in a 5′-3′ direction from a single-stranded template. Most native DNA polymerases, however, are polyfunctional. In addition to their strand synthesis activity, many DNA polymerases are able to remove nucleotides sequentially from either end of the strand (5′ exonuclease and/or 3′ exonuclease activities).

Molecular biologists have based an incredible number of laboratory applications on the multiple activities of DNA polymerase.

In April 1983, Kary Mullis of Cetus Corporation took a drive on a moonlit California road and came up with the simplest, most elegant application of all. During that drive, he conceived the basic idea for the Polymerase Chain Reaction (PCR), which is a technique for amplifying a specific target DNA sequence *in vitro*. Ten years later, Dr. Mullis received the 1993 Nobel Prize in Chemistry, underscoring the importance of that one simple idea.

### The PCR Cycle

PCR amplification can turn a few molecules of a specific target nucleic acid (too little to be analyzed directly or used in biochemical reactions) into as much as a microgram of DNA.

PCR is closely patterned after the natural DNA replication process (Saiki et al., 1985). Two oligonucleotide primers flank and define the target sequence to be amplified. These primers hybridize to opposite strands of the DNA to serve as initiation points for the synthesis of new DNA strands. A thermostable DNA polymerase, such as Taq DNA Polymerase, catalyzes this synthesis.

Each round of PCR synthesis involves three steps: denaturation, annealing and extension. This three-step “PCR cycle” is repeated (Figure 1.1) several times (Mullis and Faloona, 1987).

The repetitive nature of the PCR process is the key to its amplifying power. Because the primer extension products synthesized in a given cycle can serve as templates in the next cycle, the number of target DNA copies approximately doubles every cycle. Thus, in only 20 cycles, PCR can produce about a million ( $2^{20}$ ) copies of the target.

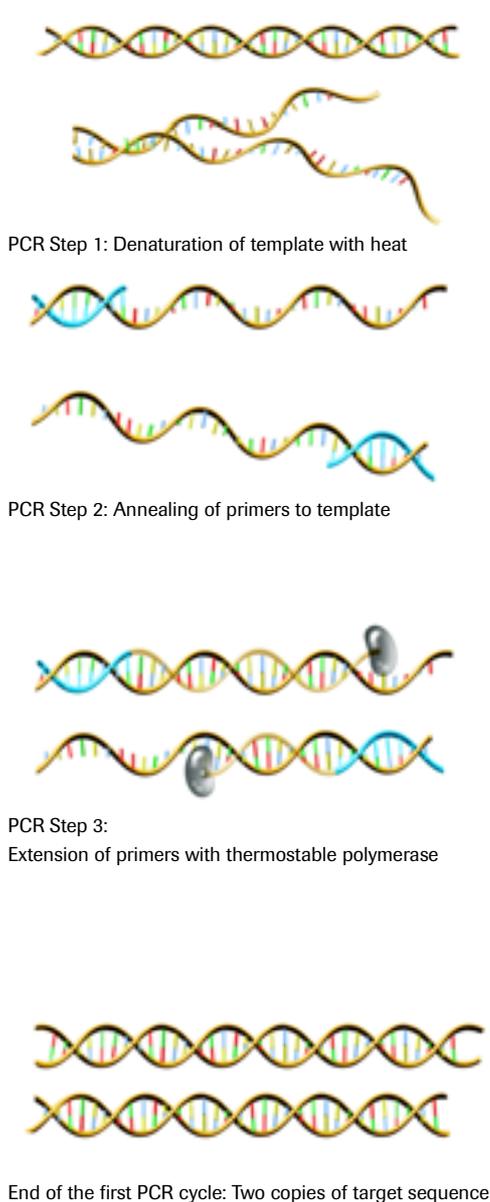
### RT-PCR

In 1987, Powell et al. described a technique that extended the power of PCR to the amplification of RNA. This technique, RT-PCR, used a reverse transcriptase to convert the rare RNA into a cDNA, then used a thermostable DNA polymerase to amplify the cDNA to detectable levels. This technique made it possible to use PCR to detect and analyze rare mRNA transcripts and other RNAs present in low abundance.



For more information on RT-PCR, see Chapter 5 in this manual.





**Step 1:** Heat (usually  $>90^{\circ}\text{C}$ ) separates double-stranded DNA into two single strands. Since the hydrogen bonds linking the bases to one another are weak, they break at high temperatures, whereas the bonds between deoxyribose and phosphates, which are stronger covalent bonds, remain intact.

This process takes place in a thermal cycler, an instrument that automatically controls the alternating cycles of heating and cooling required for PCR.

**Step 2:** The goal is not to replicate the entire strand of DNA but to replicate a target sequence of approximately 100-35,000 base pairs that is unique to the organism. Primers define the ends of that target sequence.

Primers are short, synthetic sequences of single-stranded DNA typically consisting of 20-30 bases. [Theoretically, a 16-mer is long enough to represent all unique primer sequences ( $4^{16}$ ) in a random sequence of 3 billion base pairs.]

Annealing usually takes place between  $40^{\circ}\text{C}$  and  $65^{\circ}\text{C}$ , depending on the length and sequence of the primers. This allows the primers to anneal specifically to the target sequence.

**Step 3:** Once the primers anneal to the complementary DNA sequences, the temperature is raised to approximately  $72^{\circ}\text{C}$  and a thermostable polymerase (*e.g.*, Taq DNA Polymerase) begins to synthesize new double-stranded DNA molecules which are identical to the original target DNA. It does this by facilitating the binding and joining of complementary nucleotides that are free in solution (dNTPs).

Synthesis always begins at the 3' end of the primer and proceeds exclusively in the 5' to 3' direction. Thus, the new synthesis effectively extends the primers, creating a complementary, double-stranded molecule from a single-stranded template.

**End of cycle:** At the end of the first PCR cycle, there are now two new DNA strands identical to the original target.

Note, however, that the DNA polymerase does not recognize the end of the target sequence. The newly formed strands have a beginning, which is precisely defined by the 5' end of the primer, but not a precisely defined 3' end.

Each subsequent cycle repeats and multiplies this copying process. However, as the number of cycles increases, a strand with more defined length frequently serves as the template for the newly synthesized sequence. The DNA strand synthesized from such a template then has a precisely defined length that is limited at either end by the 5' end of each of the two primers. These DNA strands are called an amplicon.

After only a few cycles, DNA strands which correspond to the target sequence are present in much larger numbers than the variable length sequences. In other words the sequence flanked or defined by the two primers is the section that is amplified.

After the appropriate number of PCR cycles (usually between 30 and 40 cycles), this repetitive, sequential process exponentially generates up to a billion of copies of the target, all within just a few hours.

**Figure 1.1.** The PCR Cycle. PCR is a cyclic DNA amplification process. Each cycle involves three steps, which are described in detail above. When the cycle is repeated several times, the net result is a rapid increase in the total number of copies of the target DNA.

## 1.2 The Evolution of PCR

The earliest version of PCR was certainly revolutionary and important, but it had some serious limitations (McPherson and Møller, 2000). Initially, the DNA was amplified with the Klenow Fragment of DNA polymerase I. However, that enzyme was not stable at high temperatures. Thus, the Klenow in the tube was inactivated during the denaturation step of each PCR cycle, and the experimenter had to add more Klenow before each extension step. Moreover, the extension had to be performed at a relatively low temperature (37°C). That made the technique boring for the scientist (because addition of Klenow was monotonous and repetitive), expensive (because a large amount of Klenow was required) and likely to introduce nonspecific products (since the primers could bind to nontarget regions of DNA at 37°C, resulting in amplification of these regions). The technique was also susceptible to outside contamination (since the tube had to be opened repeatedly to add new enzyme). Lastly, scientists could only handle a few samples at a time and, since they had to be present throughout the reaction, could not easily automate the PCR process.

Clearly, scientists solved all those limitations over time to develop the modern version of PCR, which has become an indispensable tool in almost every laboratory. The evolution of the PCR technique is mainly a story of enzymes, equipment and enhanced applications. Roche Applied Science (RAS) as well as other divisions, have made key contributions to the story in each of these areas. The following sections provide a brief overview of some of these contributions.

### PCR Enzymes

The potential of the PCR technique increased dramatically when scientists identified a thermostable DNA polymerase, Taq DNA Polymerase, that was optimally active and stable at the high elongation temperature (around 72°C) used in PCR. Because the Taq enzyme was stable during the repeated PCR cycles, researchers no longer had to interrupt the PCR process to add fresh enzyme (Saiki et al., 1988).





**1989**

**The native Taq enzyme was purified in 1986 by David Gelfand and Susanne Stoffel (who were then employed by Cetus Corporation, but later assumed important roles at Roche Molecular Diagnostics). Boehringer Mannheim (now Roche Applied Science) was one of the companies that provided a convenient recombinant version of this important enzyme (in 1989).**

Taq DNA polymerase still had drawbacks however. One such drawback was that it lacked a proofreading activity that could correct the occasional transcriptional errors (and potential mutations) that occurred during amplification. For many applications, these occasional mutations were of little concern. However, in some applications (e.g., the amplification of genomic products for sequencing and the study of allelic polymorphisms), any transcriptional errors can lead to misleading results. The commercial availability of thermostable enzymes that had proofreading activity solved this problem, providing a high fidelity version of PCR for those applications that required very accurate transcription.

**1994**

**A thermostable, proofreading enzyme, Pwo DNA Polymerase, was introduced by Boehringer Mannheim (later Roche Applied Science) in 1994.**

Another approach to solving the accuracy problem was to combine Taq DNA polymerase with a thermostable enzyme (such as Tgo DNA polymerase) or other protein that had proofreading activity.

**1995**

**One such enzyme blend, the Expand High Fidelity PCR System, was introduced by Boehringer Mannheim (later Roche Applied Science) in 1995.**

Replacing a single enzyme with an enzyme blend also led to another important advance in the evolution of PCR, the ability to amplify much longer targets (Barnes, 1994).

**1996**

**By skillfully purifying and blending enzymes, then pairing the blend with carefully optimized reaction components, Roche Applied Science was able to offer one enzyme blend (Expand Long Template PCR System, introduced in 1994) that could amplify up to 20 kb targets and another (Expand 20 kb<sup>PLUS</sup> PCR System, introduced in 1996) that could amplify up to 35 kb targets.**

By modifying Taq DNA polymerase so it was inactive at room temperature, but readily activated at DNA denaturing temperatures, researchers made possible a “hot start” version of PCR that minimized the formation of troublesome primer dimers (Birch et al, 1996).

**2000**

**Roche Applied Science introduced (in 2000) FastStart Taq DNA Polymerase for “hot start” applications. By including FastStart Taq DNA Polymerase in an enzyme blend (FastStart High Fidelity PCR System, introduced in 2003), Roche Applied Science created a high fidelity hot start PCR system that can be used in such demanding applications as multiplex PCR.**

## PCR Equipment

In early PCR experiments, researchers had to rely on a series of water baths to maintain the different temperatures required by the procedure. “Cycling” involved manual transfer of samples from one water bath to another at specified times.

In 1988, Perkin-Elmer introduced the thermal block cycler, a revolutionary device that automatically and repetitively raised and lowered the temperature of the samples during the PCR cycles. This allowed the PCR technique to be automated. Subsequent refinements of this device extended the flexibility and accuracy of PCR.

In 1991, Holland et al. described a technique (5' nuclease assay) to simultaneously amplify and detect specific DNA sequences with a fluorescent DNA-binding dye. This technique (a 5' nuclease assay) uses so called hydrolysis probes and similar FRET (fluorescence resonance energy transfer)-based techniques made it possible to analyze PCR products as they were being formed (so-called “kinetic” or “real-time” PCR analysis).



*For more information on real-time PCR, see Chapter 7 in this manual.*

In 1997, Boehringer Mannheim (later Roche Applied Science) licensed the LightCycler<sup>®</sup> Instrument technology from Idaho Technology to take advantage of these real-time PCR techniques. By using smaller samples and improved analytical software, the LightCycler<sup>®</sup> Instrument greatly reduced the time required for PCR amplification and identification of products.

Eventually, real-time PCR devices became sophisticated enough to rapidly quantify specific DNA sequences in multiple samples. This allows laboratories to use PCR for high-throughput screening (*e.g.*, for gene expression or genetic variation analyses) or to develop diagnostic PCR tests (*e.g.*, for clinical or forensic purposes). Roche Applied Science is a major supplier of reagents and instrumentation for such research. The latest such Roche Applied Science instrument, the LightCycler<sup>®</sup> 480 Real-Time PCR System (introduced in 2005), can automatically analyze up to 384 samples in a single run.





## PCR Applications

Because of PCR, “insufficient nucleic acid” is no longer a limitation in research and many medical diagnostic procedures. Most importantly, innovative researchers have continually updated and expanded the definition of “PCR applications,” increasing the usefulness and scope of the technique. Even a brief review of these innovative applications is beyond the scope of this article. However, here is just a random sampling of PCR applications that are currently important:

- ▶ Automated PCR instruments that permit real-time detection and analysis of many products in a single run (*e.g.*, the LightCycler® Carousel-based System, the LightCycler® 480 Real-Time PCR System, and the COBAS® TaqMan® 48 Analyzer).
- ▶ Clonal amplification of genomic DNA in an emulsion (emPCR) to make ultrarapid sequencing of the genome possible (*e.g.*, in the Genome Sequencer 20 System).
- ▶ Introduction of molecular “tags” (such as biotin and digoxigenin) into the PCR product during amplification, allowing these products to be used as sensitive hybridization probes in medical diagnostic tests (*e.g.*, in the COBAS® AMPLICOR® Analyzers).
- ▶ Simultaneous amplification of multiple sequences in a single sample (multiplex PCR) (*e.g.*, for identification of human single nucleotide polymorphisms in genomic DNA).
- ▶ Studies of genetic variability (*e.g.*, to determine the genetic basis of diseases).
- ▶ Amplification of DNA for identity testing (*e.g.*, DNA fingerprinting).
- ▶ Study of epigenetic mechanisms (such as DNA methylation, histone acetylation, and RNA interference) involved in activation and inactivation of genes (*e.g.*, addition of T7 promoters to DNA templates to allow generation of double-stranded RNA for gene knockdown studies).
- ▶ Creation of novel DNAs by *in vitro* mutagenesis.
- ▶ Exploration of evolutionary relationships via examination of ancient DNA from fossils.



*Each of these applications have led to many more published articles than can be mentioned here.*

## 1.3 Purpose of this PCR Applications Manual

PCR has come a long way, but we still feel there is a need for a basic PCR handbook such as this PCR Applications Manual. This 3<sup>rd</sup> edition of the manual repeats, extends and updates information contained in the previous two editions, including:

- ▶ General guidelines for setting up a PCR lab (chapter 2).
- ▶ Suggestions for optimizing the production of primers and templates (chapter 3).
- ▶ A basic set of PCR (chapter 4) and RT-PCR (chapter 5) protocols, including tips on how to get the best results with our products.
- ▶ Suggestions for cleaning up, analyzing and cloning PCR products (chapter 6).
- ▶ Guidelines to help you decide what PCR technique or reagent to use (protocol and product guides in chapters 3 - 6 and the Appendix).
- ▶ A brief look at some of the current applications for PCR and RT-PCR, which take advantage of products from Roche Applied Science (chapter 7).
- ▶ Applications from papers submitted to Roche Applied Science, each describing an application for one or more of the PCR and RT-PCR products described in previous chapters (chapter 8).
- ▶ A PCR toolbox that summarizes a wealth of technical information, including troubleshooting tips, useful PCR parameters, and detailed ordering information for all the products mentioned in this manual (Appendix).

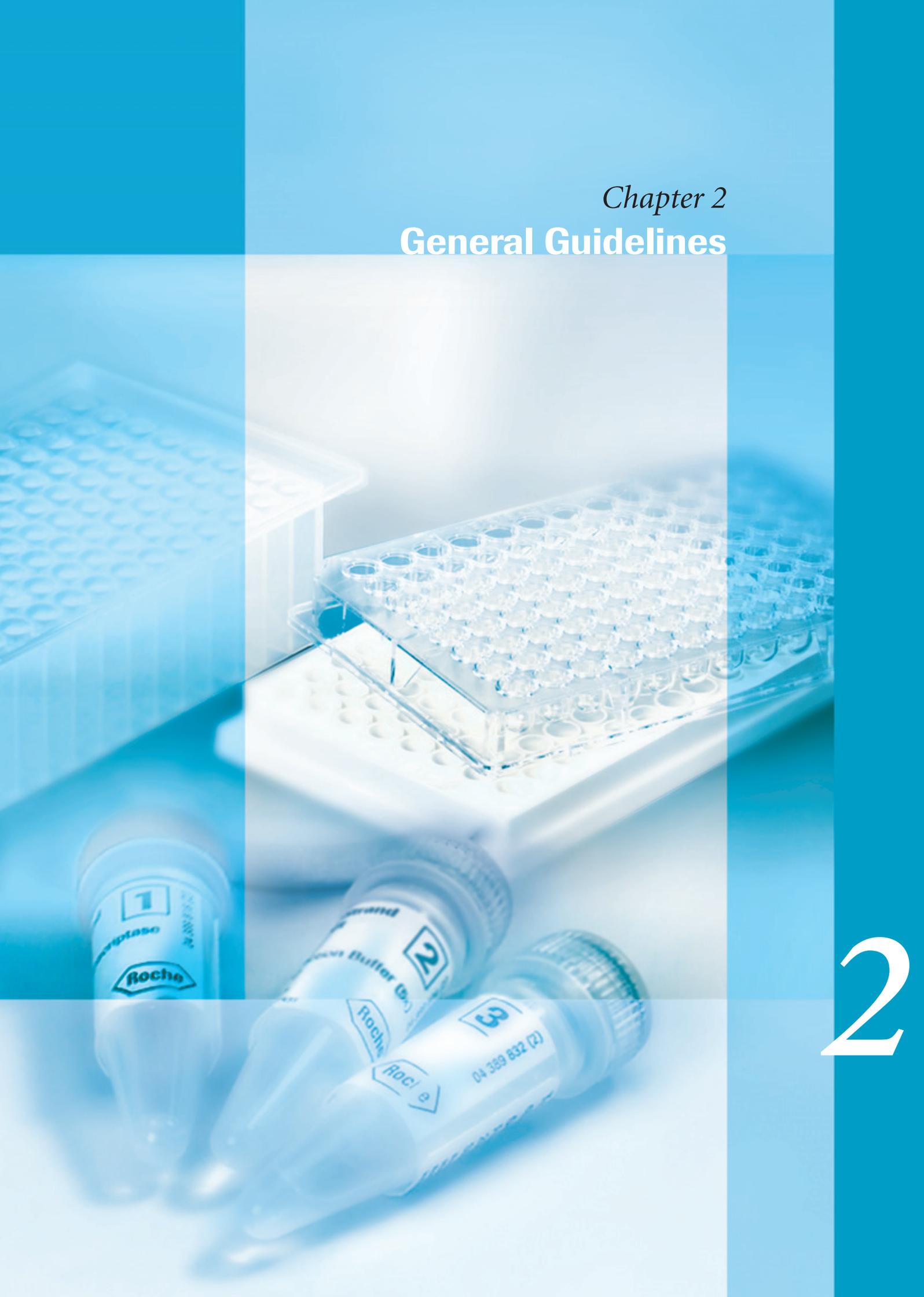
We hope that this information will be useful to both those new to PCR and those with a great deal of experience with the technique.



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# 1

*Chapter 2*  
**General Guidelines**



2	General Guidelines	Page
2.1	Preventing Contamination in the PCR Laboratory .....	19
2.1.1	Setting Up the PCR Laboratory Space Correctly .....	20
2.1.2	Cultivating Laboratory Habits That Prevent Cross-Contamination .....	21
2.1.3	Using Uracil N-Glycosylase To Eliminate Carryover Contamination .....	26
2.2	Factors To Consider When Setting Up a PCR Laboratory .....	27
2.2.1	Equipment Required.....	27
2.2.2	Choosing the Correct Enzymes for PCR and RT-PCR.....	29
2.2.3	Other Important Reaction Components.....	33
2.2.3.1	Templates and Primers .....	33
2.2.3.2	Nucleotides and Magnesium Ions .....	33
2.2.3.3	Other Reaction Components.....	35
2.2.4	How Cycling Parameters Affect a PCR.....	36
2.3	Typical Workflow for PCR/RT-PCR.....	37



## 2. General Guidelines

### 2.1 Preventing Contamination in the PCR Laboratory

PCR generates large numbers of amplicons. Therefore, it carries a high risk of contamination. Even infinitesimal amounts of contaminating DNA may lead to significant concentrations of amplicons and false results.

Clearly, contaminant-free assays are critical for identifying pathogenic organisms or “fingerprinting” DNA. Any PCR lab involved in developing such assays must avoid contamination at all costs since their assay samples may be hazardous to the health of laboratory personnel. In addition, these assays, if ultimately approved for clinical or forensic use, will affect the lives of many people.

Similarly, labs that use PCR for exploring the behavior and function of genes must take steps to avoid contamination, since no one wants to waste enormous amounts of time and money pursuing research based on contaminated assays.

#### Sources of Contamination

Contamination may be defined as “accidental contact or mixing of the sample with exogenous material (nucleic acids or microbial organisms) that make the sample impure or corrupt.” As stated above, any source of contamination, no matter how small, can lead to false positive results.

Contamination usually arises from two sources:

- ▶ Cross-contamination, or contamination of the sample with non-amplified material from the environment (*e.g.*, aerosols, cloned DNA molecules that carry the target gene) or other samples. This may be due to parallel storage, handling or processing of intermediate samples.
- ▶ End-product (*i.e.*, carryover) contamination, or contamination of the sample with amplicons from earlier PCRs, leading to subsequent amplification of both target and contaminant amplicons. This may be due to storage, handling or processing of samples between PCRs.

In sections 2.1.1 and 2.1.2 below, we will describe laboratory practices designed to exclude cross-contamination from exogenous microbial (*i.e.*, bacterial, fungal or viral) DNA in routine diagnostic laboratories. These practices are currently used in our Application Laboratory for the LightCycler® 2.0 System (Penzberg, Germany). Although developed for real-time PCR assays on a LightCycler® 2.0 Instrument, these guidelines are also generally suitable for (or can be adapted to) other applications, such as qualitative PCR analysis on a block cycler in a research laboratory.



*These practices are based on working procedures that routine diagnostic laboratories must follow to comply with German regulations. These regulations cover, among other things, the physical separation of areas for extraction, PCR setup and amplification/detection, and the use of filtered tips. For a more detailed discussion, see Mauch et al. (2001).*

A technique for preventing carryover contamination will be discussed in section 2.1.3 below.

## 2.1.1 Setting Up the PCR Laboratory Space Correctly

Contaminant DNA usually finds its way into PCR assays through:

- ▶ Working environment (e.g., rooms and equipment, lab benches and other work surfaces).
- ▶ Consumable reagents and supplies (e.g., oligonucleotides, media for sample collection and transport, plasticware).
- ▶ Laboratory staff and their work habits (e.g., contaminants from skin, hair, gloves, production of aerosols during pipetting).

Therefore, preventing microbial cross-contamination has two equally important components: setting up laboratory space correctly (discussed below) and cultivating laboratory habits that prevent cross-contamination (discussed in section 2.1.2). Once the lab is correctly configured and good laboratory habits are followed by all personnel, preventing contamination become almost automatic and the laboratory can focus on producing meaningful results.

Usually, cross-contamination and environmental contamination occur during sample manipulations. So, from the very beginning, the PCR laboratory should be set up to accomplish two goals:

- ▶ Physically separating the lab areas used for sample preparation, DNA extraction, amplification and post-PCR analysis, and
- ▶ Minimizing the number of sample manipulations.

### Physical Separation of Work Areas

In our laboratory, separate areas are provided for preparation of samples for nucleic acid isolation, automated nucleic acid isolation/processing in the MagNA Pure LC Instrument and amplification/analysis in the LightCycler® Instrument.

Even if all steps are performed manually, a properly set-up PCR lab will contain physically separate areas for sample preparation, DNA extraction, amplification and post-PCR analysis. Ideally, each of these takes place in a separate room and each of these rooms has:

- ▶ A dedicated set of reagents, pipettes, and disposables that are kept and used exclusively in that room.

 *For analysis of microbiological samples, we recommend storing bags that contain disposables (tubes, tips, reagent tubs) in a separate, suitable room. (Presence of UV light fixtures in this storage room is recommended.)*

- ▶ A full set of equipment, such as refrigerator, centrifuges and safety cabinets.
- ▶ A dedicated set of lab coats that are kept and used exclusively in that room.
- ▶ A safety cabinet, if samples are potentially hazardous or infectious.

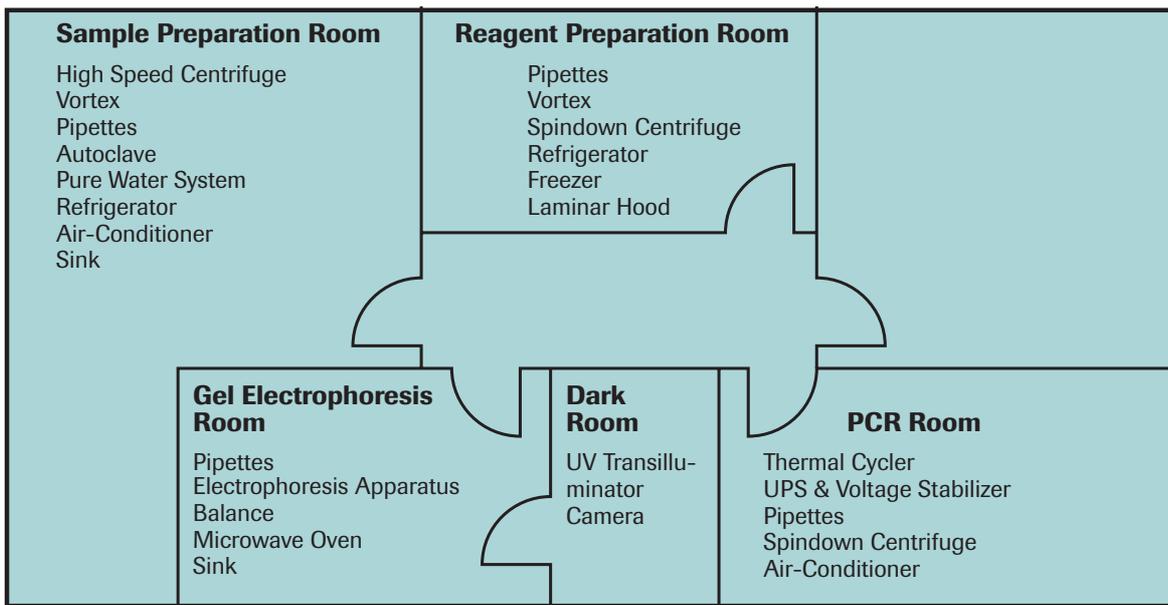


Figure 2.1.1 Organization of a PCR laboratory with separate pre- and post-PCR rooms.

### Minimizing Sample Manipulation

The best way to minimize sample manipulation (and the corresponding risk of contamination) is to automate as many steps in PCR analysis as possible. See “Guidelines for Preventing Cross-Contamination” in section 2.1.2 below for more information on what steps can be automated.

## 2.1.2 Cultivating Laboratory Habits That Prevent Cross-Contamination

By itself, a properly set-up PCR laboratory space will not prevent cross-contamination. Once people start working in that space, their actions introduce significant risks of contaminating the PCR samples.

Therefore, preventing cross-contamination requires the cooperation of all laboratory personnel. Only if all the people in the laboratory follow good work habits, will the laboratory be free of contamination.

This may seem unduly harsh. We all know laboratory personalities that seem to lead a charmed life, working with total disregard of good laboratory practices yet seeming to always get good results. However, once those persons join a laboratory group, their actions have the potential to ruin the results of all their co-workers. That means even if you are tempted to take short cuts in a PCR procedure, or to ignore the precautions outlined below, don't do it!

*Example:* Never reverse the direction of the workflow (e.g., by transporting amplified material into the DNA extraction room). The steps in the PCR workflow are always unidirectional, from DNA extraction to amplification. This principle holds for working procedures as well as for reagents and consumables.

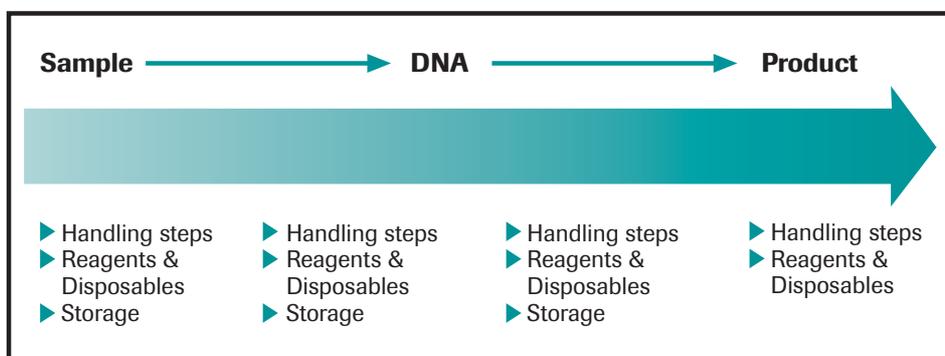
Below we list a general strategy for identifying sources of contamination and developing guidelines to avoid them.

## Developing Laboratory Work Guidelines: Assessing and Avoiding Risks in PCR Workflow

Even in the best physical workspaces, microbial DNA contamination can still get into PCR samples. Therefore, in developing good laboratory habits, start by attempting to quantify the hazards and risks a worker may encounter during the entire experimental procedure. A contamination hazard may be defined as the introduction of contaminating nucleic acid from any possible source; risk is the probability that the hazard will occur.

The goal of a risk assessment is to carefully consider all hazards and risks associated with the whole process, so that the risk can be minimized and preventive actions can be implemented. Any change in the process (*e.g.*, new lab personnel, new equipment, a new supplier of consumables or a change in assay design) requires a new risk assessment.

For risk assessment, consider sample handling steps, materials (reagents and disposables) used, and storage of intermediate products (such as extracted DNA) (Figure 2.1.2).



**Figure 2.1.2. Critical steps in PCR workflow.**

Once risks have been identified and quantified, the laboratory can adapt strategies to minimize or avoid these risks. These strategies can be formalized as laboratory work guidelines.

For example, we list below one possible source of contamination and the strategies we adapted to avoid these risks.

**!** *Because each laboratory has unique practices and environments, no single strategy for risk assessment and avoidance can be appropriate for all laboratories. Each laboratory should perform its own risk assessment and adapt avoidance strategies that are appropriate for the risk.*

### Strategies for Avoiding One Possible Source of Contamination

Most manufacturers of consumables and reagents do not guarantee that their products are DNA-free, although most would guarantee them to be sterile. Consequently, these products may constitute a risk of contamination.

The problem arises because in microbiological terms, “sterile” means “absence of viable, replicable bacteria or other microorganism.” Thus, “sterile” does not imply absence of bacterial or fungal DNA in any form. In fact, commercially available “sterile” instruments and culture media may frequently contain microbial DNA. The instruments and media are still “sterile” because the source of this DNA (= the organism) cannot be cultivated. The amount of such contamination depends on the supplier as well as the production lot.

Generally, any reagent, device or consumable has the potential to cause false positive results because it may be contaminated with microbial DNA. Specifically, most molecular biology enzymes contain some amount of bacterial DNA since they were derived from different strains of bacteria.

One strategy for preventing such false positives is to screen individual reagents and consumables in PCR before they are used to assay unknowns. This is especially true for oligonucleotides (primers and probes).

In addition, you should include a negative control in the PCR run for every step that uses fresh reagents and disposables. It is also helpful to include a reliable positive control to demonstrate true gene expression.

### Guidelines for Preventing Cross-Contamination

The tables below summarize the guidelines that our laboratory follows to prevent PCR contamination.



*If each member of the laboratory converts these guidelines into personal work habits, most contamination can be avoided and, if contamination occurs, its effects can be minimized by identification and elimination of the contamination source.*

# 2

Area of Concern	Steps To Take
All stages	
Disposables and reagents	<ul style="list-style-type: none"> <li>▶ Use of reagents and disposables is room-specific. Always store a unique set of reagents and disposables in each of the procedure-specific rooms (DNA isolation, PCR, etc.) and use that set only in that room.</li> <li>▶ Always use the highest quality of reagents available for each step of the procedure.</li> <li>▶ To guarantee that bags of plastic disposables remain free of DNase and RNase, reseal these bags immediately after removing the disposables needed for each procedure.</li> <li>▶ Use each plastic disposable only once.</li> <li>▶ Divide reagents into aliquots that will be consumed in a single procedure (DNA isolation, PCR, etc.). Make only enough reagents for the number of samples you are preparing (e.g., if you usually run 10 samples, make aliquots with enough reagents for 11 reactions).</li> <li>▶ Minimize pipetting steps by using reagent premixes (e.g., PCR master mixes available from Roche Applied Science) whenever possible.</li> <li>▶ Discard any reagents left over from a procedure rather than storing them and reusing them.</li> <li>▶ Always use pipette tips that prevent aerosol formation.</li> <li>▶ Regularly wipe pipette tips with ethanol-soaked tissue.</li> </ul> <p> <i>Do not get any ethanol into the pipette tip itself.</i></p>
Cleaning/Decontaminating the laboratory environment	<ul style="list-style-type: none"> <li>▶ Access to the laboratory is controlled and limited.</li> <li>▶ Develop a formal written plan for monitoring cleaning and decontaminating the laboratory.<sup>9</sup> Make this plan comprehensive (e.g., include such matters as how and where to clean laboratory coats.)</li> <li>▶ Train all laboratory and cleaning personnel in routine cleaning and decontamination procedures.</li> <li>▶ Always use cleaning agents that are suitable for and dedicated to decontaminating PCR lab surfaces and instruments. (Several decontaminating reagents are commercially available, e.g., License-to-Kill, DNAzap, DNA Remover, or DNA-ExitusPlus. Alternatively you may also use bleach [10% solution of sodium hypochloride]; before using bleach on instrument surfaces make sure this is allowed for your instrument.) Do not use reagents that are used to clean other parts of the facility.</li> <li>▶ UV irradiation (at 254 nm) can “inactivate” DNA contamination in disposable, reagents and PCR samples. UV dimerizes the thymidine residues in DNA, thus rendering it incapable of serving as a PCR template. Suitable irradiation devices include those for crosslinking DNA to nylon membranes.</li> </ul> <p> <i>Be sure to UV irradiate PCR samples <u>before</u> adding the template DNA, DNA polymerase, and dNTPs!</i></p>
Water	<ul style="list-style-type: none"> <li>▶ Always use ultrapure water for reagent and sample dilution.</li> <li>▶ Do not use autoclaved water because it may be contaminated with DNA if the autoclave is also used for sterilization of cultures.</li> <li>▶ Since DNA is highly soluble in water, water may be used to clean the laboratory environment, dilute DNA (to minimize the risk of contamination) and to transport DNA away from contaminated benches.</li> <li>▶ Autoclaving facilities must be completely separate from the PCR labs and steam must not be carried into the PCR labs via the air conditioning system.</li> </ul> <p> <i>Since DNA is soluble in water, it is also soluble in steam. Therefore, steam which escapes from an autoclave (e.g., after sterilization of cultures) may contain large amounts of DNA.</i></p>
Laboratory clothing	<ul style="list-style-type: none"> <li>▶ Always wear gloves and lab coats during the procedures.</li> <li>▶ Always clean lab clothing according to the formal laboratory cleaning plan. (See above.)</li> </ul>

Area of Concern	Steps To Take
<b>Preparing reagents and samples for DNA isolation</b>	
General	Follow all guidelines under “All stages” above.
Physical space	Always dedicate separate rooms in the laboratory that are used only for preparing reagents and samples for DNA isolation.
Handling of biohazardous or infectious material	<ul style="list-style-type: none"> <li>▶ Prepare all sample materials and positive controls in a safety cabinet.</li> <li>▶ Follow all country-specific guidelines and regulations for handling infectious or biohazardous material.</li> </ul>
<b>DNA isolation</b>	
General	Follow all guidelines under “All stages” above.
Physical space	Always dedicate a separate room in the laboratory that is used only for DNA isolation.
Isolation procedure	<ul style="list-style-type: none"> <li>▶ Always use reagents that are specifically designed for isolation of DNA.</li> <li>▶ Ideally, automate as much of the nucleic acid isolation procedure as possible by using an automated instrument (e.g., the MagNA Pure LC Instrument* or the MagNA Pure Compact Instrument*)</li> <li>▶ At the very least, minimize the number of hands-on steps required by using manual nucleic acid isolation kits.*</li> </ul> <p> <i>Starting with the nucleic acid extraction step, manual PCR steps will always carry a higher risk of contamination than the corresponding automated steps.</i></p>
Preparation of instrument (e.g., MagNA Pure LC Instrument) for automated DNA isolation (optional)	<ul style="list-style-type: none"> <li>▶ Decontaminate the instrument with UV light for 30 minutes.</li> <li>▶ Change all waste collectors (e.g., on the MagNA Pure LC Instrument, the dropcatcher, the waste bag on the waste slide, the waste bottle tray, the waste bottle, and the waste funnel.)</li> <li>▶ If using biohazardous material, set the instrument to automatically discard waste.</li> <li>▶ After the isolation run, make the instrument ready for the next run by performing the following cleaning steps: <ul style="list-style-type: none"> <li>▶ Seal and autoclave all waste receptacles (e.g., on the MagNA Pure LC Instrument, the waste bags and the waste bottle).</li> <li>▶ On the MagNA Pure LC Instrument, decontaminate all waste collectors and accessories (e.g., the cooling block) as recommended by the manufacturer.</li> </ul> </li> </ul>
<b>PCR</b>	
General	Follow all guidelines under “All stages” above.
Physical space	Always dedicate a separate room in the laboratory that is used only for PCR.
PCR set-up	<ul style="list-style-type: none"> <li>▶ If your thermal cycler requires a mineral oil overlay, make sure the mineral oil does not become contaminated by careless pipetting. For example, pour an aliquot of mineral oil from a stock bottle to a microfuge tube, use the oil in this tube for dispensing to sample tubes, then discard the microfuge tube at the end of the set-up.</li> <li>▶ Perform all set-up procedures that require open reagents and boxes of disposables in a PCR cabinet with a laminar (nonturbulent) airflow.</li> <li>▶ Prepare positive controls in a room separate from the sample preparation room.</li> <li>▶ If possible, to minimize hands-on steps, use an instrument that automatically performs the PCR sample set-up (e.g., the MagNA Pure LC Instrument).</li> </ul>
Cooling blocks	<ul style="list-style-type: none"> <li>▶ Cooling blocks that are used in different parts of the laboratory should only be opened in a PCR cabinet with laminar airflow.</li> <li>▶ After each use, clean the cooling blocks with approved reagents.</li> <li>▶ In addition to the above cleaning, completely degrade all nucleic acids on the block by cleaning the block with DNAZap; completely remove RNase contamination by cleaning with RNaseZap.</li> </ul>
Controls	<ul style="list-style-type: none"> <li>▶ Always prepare positive and negative control samples for every PCR run.</li> <li>▶ Include a negative control in a PCR run for every step that uses fresh reagents and disposables (e.g., sample preparation, DNA extraction and amplification). For example, if you isolate sample material from a culture, include one sample that contains just culture medium.</li> <li>▶ Prepare positive controls in a room separate from the sample preparation room.</li> <li>▶ Optionally, include an internal (or endogenous) positive control in the run to help identify PCR inhibitors.</li> </ul>

Area of Concern	Steps To Take
PCR continued	
PCR sample tubes	<ul style="list-style-type: none"> <li>▶ Use sterile, DNA-free, disposable forceps to close the tube lids.</li> <li>▶ Never touch any surface of the disposable tubes (<i>e.g.</i>, interior of lids) that will make contact with the sample.</li> </ul>
Automation of PCR	A real-time PCR instrument ( <i>e.g.</i> , the LightCycler <sup>®</sup> 2.0 Instrument* or the LightCycler <sup>®</sup> 480 Instrument) can automate the amplification process.
<b>Post-PCR processing</b>	
General	Follow all guidelines under “All stages” above.
Physical space	Always dedicate a separate room in the laboratory that is used only for post-PCR processing of PCR products.
Automation of product detection	A real-time PCR instrument ( <i>e.g.</i> , the LightCycler <sup>®</sup> 2.0 Instrument*) can automate the product detection process.

- <sup>a)</sup> For example, our laboratory follows the country-specific regulations for setting up a laboratory hygiene plan. In Germany, such regulations are available from the Robert Koch Insitut.
- \* Manual nucleic acid isolation kits, the MagNA Pure LC Instrument and the LightCycler<sup>®</sup> 2.0 Instrument are available from Roche Applied Science.

### 2.1.3 Using Uracil N-Glycosylase To Eliminate Carryover Contamination

Another serious source of contamination is carryover of product from a previous PCR. This is a particularly serious problem in laboratories that amplify the same target in many different samples. Even minute amounts of carryover can lead to false positive results. For example, Kwok and Higuchi (1989) calculated that, if 100 µl of a PCR product from a typical PCR were diluted into an olympic-size swimming pool and mixed, a 100 µl sample taken randomly from that pool-sized mixture would contain approximately 400 molecules of DNA.

Obviously, the guidelines listed in section 2.1.2 above can help prevent carryover contamination as well as cross-contamination. However, there is another precaution that can be taken to specifically negate the effects of carryover contaminants (*i.e.*, contamination with previously amplified PCR products).

Including uracil-DNA N-glycosylase (UNG) and deoxyuridine triphosphate (dUTP) in all PCRs can help prevent carryover contamination. The prevention procedure involves incorporating dUTP during PCR so the amplicons contain deoxyuracil (dU), then pretreating subsequent PCR mixtures with UNG. If a dU-containing contaminant from a previous PCR is present in a new PCR, it will be cleaved by a combination of UNG digestion and the high temperature of the initial denaturation step; after treatment, it cannot serve as a PCR template. Since any newly added DNA template (*e.g.*, your target DNA) contains thymidine rather than uridine, it is not affected by this procedure. For more information on the use of UNG, see section 4.6, “Preventing Carryover” in Chapter 4.



*Country-specific safety and health regulations also contain many procedures for minimizing carryover contamination. Although this manual does not discuss these regulations, we recommend implementation of such procedures and compliance with all such guidelines.*

## 2.2 Factors To Consider When Setting Up a PCR

### 2.2.1 Equipment Required

Below is a brief overview of the equipment needed for PCR.

#### Thermal Cyclers

A thermal cycler must, at a minimum, accurately and reproducibly maintain the three PCR incubation temperatures (denaturation, annealing and elongation), change from one temperature to another (“ramp”) over a definable time, reach the selected temperatures without significantly over- or undershoot and cycle between the temperatures repeatedly and reproducibly.



*The thermal cycler cited most frequently in the protocols given in Chapters 4 and 5 of this manual is the Applied Biosystems GeneAmp System 2400. If you choose another thermal cycler, you will need to adjust the cycling conditions given in this Manual to obtain optimal results.*

When choosing a thermal cycler, consider the following factors to determine which best fits your experimental needs:

- ▶ What size (or size range of) sample is accommodated.
- ▶ How samples are held (e.g., in a thermal block or other device).
- ▶ How samples are heated and cooled (e.g., by air, electrical resistance within a fluid or electrical resistance modulated with a Peltier semiconductor device).
- ▶ How sample evaporation is controlled (i.e., by a heated lid on the thermal cycler or by a simple mineral oil overlay of the samples).
- ▶ How the reaction steps are programmed (e.g., determination of ramping rates, inserted pauses in the reaction).
- ▶ How temperature is monitored.
- ▶ Whether the reaction can be fully or partially automated.
- ▶ Whether the device simplifies PCR optimization (e.g., if it has a gradient feature that allows testing of several preset temperatures simultaneously on a single thermal block).
- ▶ Whether and how product accumulation can be monitored.
- ▶ Whether reaction products can be refrigerated and kept in the thermal cycler for short-term (e.g., overnight) storage.

### Mineral Oil

If the thermal cycler you are using has a heated lid, there is no need to overlay the samples.

However, if the thermal cycler lacks a heated lid, you should place a layer of mineral oil atop the reaction mixture to prevent evaporation during PCR. For best results, use a high quality light mineral oil (available from chemical companies such as Sigma-Aldrich, Inc.)

### Sample Containers

A variety of disposable sample containers is available for PCR. Some examples include:

- ▶ **Reaction tubes:** The reaction tubes used for PCR affect the rate at which heat transfers from the thermal cycler to the reaction mixture. Therefore, we recommend that you use thin-walled reaction tubes that are specifically designed for PCR and that fit precisely into the wells of your thermal cycler. Some tubes (and the corresponding caps) are available in convenient strips that fit into a row of the thermal cycler. A variety of such tubes is available from Roche Applied Science.
- ▶ **Capillaries:** For rapid PCR, small-volume capillaries allow much faster heat transfer. However, these only fit in sophisticated PCR instruments (*e.g.*, the LightCycler<sup>®</sup> 2.0 Instrument from Roche Applied Science).
- ▶ **Microplates:** For medium- and high-throughput applications, the preferred sample carrier is a PCR microplate.

### Other Plasticware and Disposables

**Tubes for reaction set-up:** For aliquoting reagents and preparing master reaction mixes (but not for the PCR itself), the most convenient container is the disposable, conventional 1.5 ml microfuge tube.

**Disposable pipette tips:** As much as possible during reaction set-up, use aerosol-resistant pipette tips, to prevent contamination of the samples.

### Equipment for Product Analysis

Most laboratories already have the equipment needed for routine analysis of PCR products, including:

- ▶ Agarose gel electrophoresis, the most common way to detect PCR products qualitatively.
- ▶ Acrylamide gel electrophoresis, for analysis of smaller PCR products.
- ▶ Southern/slot/dot blots, for quantitation and sequencing of PCR products.



*For more information on the use and suitability of various analytical techniques for PCR, see Chapter 6.*

For faster, more sophisticated analysis of PCR products (*e.g.*, for high-throughput applications), you may want to consider using real-time PCR instruments. See Chapter 7 for more information on these powerful instruments.

## 2.2.2 Choosing the Correct Enzymes for PCR and RT-PCR

The choice of a PCR enzyme can profoundly affect the outcome of the PCR. As mentioned in Chapter 1, researchers have purified, modified and commercialized a variety of PCR and RT-PCR enzymes. Below is a brief overview of the major types of enzymes available.

### Taq DNA Polymerase and FastStart Taq DNA Polymerase

The primary requirements for a DNA polymerase used in PCR are optimal activity at temperatures around 75°C and the ability to retain that activity after prolonged incubation at even higher temperatures (95°C). The first thermostable DNA polymerase to be widely used for PCR was Taq DNA Polymerase. For many conventional PCRs that do not require extensive optimization, Taq DNA Polymerase is still a good choice. High quality, recombinant Taq DNA Polymerase (such as the preparation available from Roche Applied Science) produces the best results. Nevertheless a major drawback of standard Taq DNA Polymerase is its activity at temperatures below its optimum of 72°C. In non-optimized systems, this will lead to formation of primer-dimers due to elongation of primers annealed to each other before the first DNA denaturation step has occurred.

More recently, modifications of the Taq enzyme were developed which make it more useful for PCR. These so-called “hot start” preparations of Taq DNA Polymerase (e.g., FastStart Taq DNA Polymerase available from Roche Applied Science) are inactive at low temperatures, but readily activated at DNA denaturing temperatures. Thus, hot start polymerases minimize the formation of troublesome primer-dimers during reaction set-up.

FastStart Taq DNA Polymerase is a chemically modified form of thermostable recombinant Taq DNA Polymerase that shows no activity up to 75°C. The enzyme is active only at high temperatures, where primers no longer bind non-specifically. The enzyme is completely activated (by removal of blocking groups) in a single pre-incubation step (95°C, 2-4 minutes) before cycling begins. Activation does not require the extra handling steps typical of other hot-start techniques.

Today, therefore, FastStart Taq DNA Polymerase is the best enzyme for most basic PCRs (i.e., those that amplify normal or GC-rich templates up to 3 kb in length).

### Proofreading DNA Polymerases

Standard Taq DNA Polymerase and its chemically modified form FastStart Taq DNA Polymerase lack the ability to detect the incorporation of a wrong nucleotide during polymerization and cannot remove such misincorporated nucleotides. Thus, these DNA polymerases have a relatively high PCR error rate. Furthermore, misincorporation during replication may lead to stalling of the DNA polymerase and thus to shortened PCR products. If your application requires a better fidelity than provided by (FastStart) Taq DNA Polymerase (e.g., if you want to clone or sequence a PCR product), you need to use a thermostable DNA polymerase with “editing” or “proofreading” activity. Proofreading DNA polymerases possess an additional 3'-5' exonuclease activity which removes misincorporated nucleotides.



*For details of a lacI-based assay that can determine the transcriptional accuracy (fidelity) of a PCR enzyme, see Frey and Suppmann (1995).*

Pwo SuperYield DNA Polymerase (available from Roche Applied Science in most countries, but not available in the U.S.) has a much (up to 18-fold) lower error rate than Taq DNA Polymerase, which makes this enzyme ideal for high fidelity amplification of targets with length up to 3 kb.

Information Note: A drawback of all known proofreading DNA polymerases is, that they cannot be used for carryover prevention using the Uracil-DNA N-glycosylase (UNG) method. Proofreading DNA polymerases are of archaeal origin and belong to the Family B-type DNA polymerases. All B-type DNA polymerases possess a proofreading (3'→5' exonuclease) activity but lack a 5'→3' exonuclease activity. In contrast to Taq DNA Polymerase, archaeal DNA polymerases cannot copy DNA strands containing uracil residues: they possess a "read-ahead" function that detects dU residues in the template strand and stalls DNA synthesis (Martin A. Greagg *et al.*, 1999). Thus, exchanging dTTP by dUTP would lead to inhibition of PCR when using a B-type DNA polymerase.

## PCR Enzyme Mixtures and Blends

As PCR became more sophisticated, polymerase mixtures and blends began to be used. For specific purposes, these blends actually outperformed the individual DNA polymerases. Several examples are given below.

One approach to overcoming the limited accuracy of Taq DNA Polymerase alone was to combine the Taq DNA Polymerase with a thermostable, proofreading polymerase or another protein that has proofreading activity. Such a blend (*e.g.*, the Expand High Fidelity PCR System from Roche Applied Science) transcribes DNA approximately threefold more accurately than the Taq DNA Polymerase alone and can be used for high fidelity amplification of moderately long (up to 5 kb) targets.

For amplification of GC-rich sequences with high yield, the GC-RICH PCR System\* from Roche Applied Science is the best mix to choose.

For amplification of multiple targets in a single reaction (multiplex PCR), FastStart High Fidelity PCR System offers several advantages. This enzyme mixture transcribes more accurately (up to fourfold higher fidelity) than Taq DNA Polymerase alone and is better able to amplify sequences with high (40 – 60%) GC content. Also, the blend is very sensitive, producing good yields of amplicon from small amounts of target DNA.

Certain blends of enzymes, buffers and additives allow accurate amplification of very long templates (*e.g.*, up to 25 kb targets with the Roche Applied Science Expand Long Range dNTPack).

 *To allow high-fidelity PCR in combination with prevention of carryover contamination using the Uracil DNA Glycosylase method, Roche Applied Science introduced the Expand High Fidelity<sup>PLUS</sup> PCR System and the FastStart High Fidelity PCR System: these enzyme blends consist of Taq DNA Polymerase and a novel proofreading protein, isolated and characterized by Roche Applied Science. This protein mediates proofreading activity but has no polymerase activity itself and thus PCR is not inhibited by dU-containing DNA.*

 *For a complete listing of PCR enzymes available from Roche Applied Science, see the Ordering Information in the Appendix. For more information on the properties of PCR enzymes, see the table, "Comparison of PCR Enzymes," below. For more information on the appropriateness of the different PCR enzymes for specific applications, see the PCR Protocol Selection Guide (in Chapter 4) or the PCR Selection Guide (in the Appendix).*

## Reverse Transcriptases

RT-PCR extends the power of PCR to the amplification of RNA by using an RNA-dependent DNA polymerase, commonly called a reverse transcriptase, to convert an RNA into a cDNA, then using a thermostable DNA polymerase to amplify the cDNA to detectable levels. This combination of reactions can be performed as either a one-step (consecutively, in a single tube) or a two-step (consecutively, in separate tubes) process. There are a variety of reverse transcriptases available for both one-step and two-step RT-PCR.

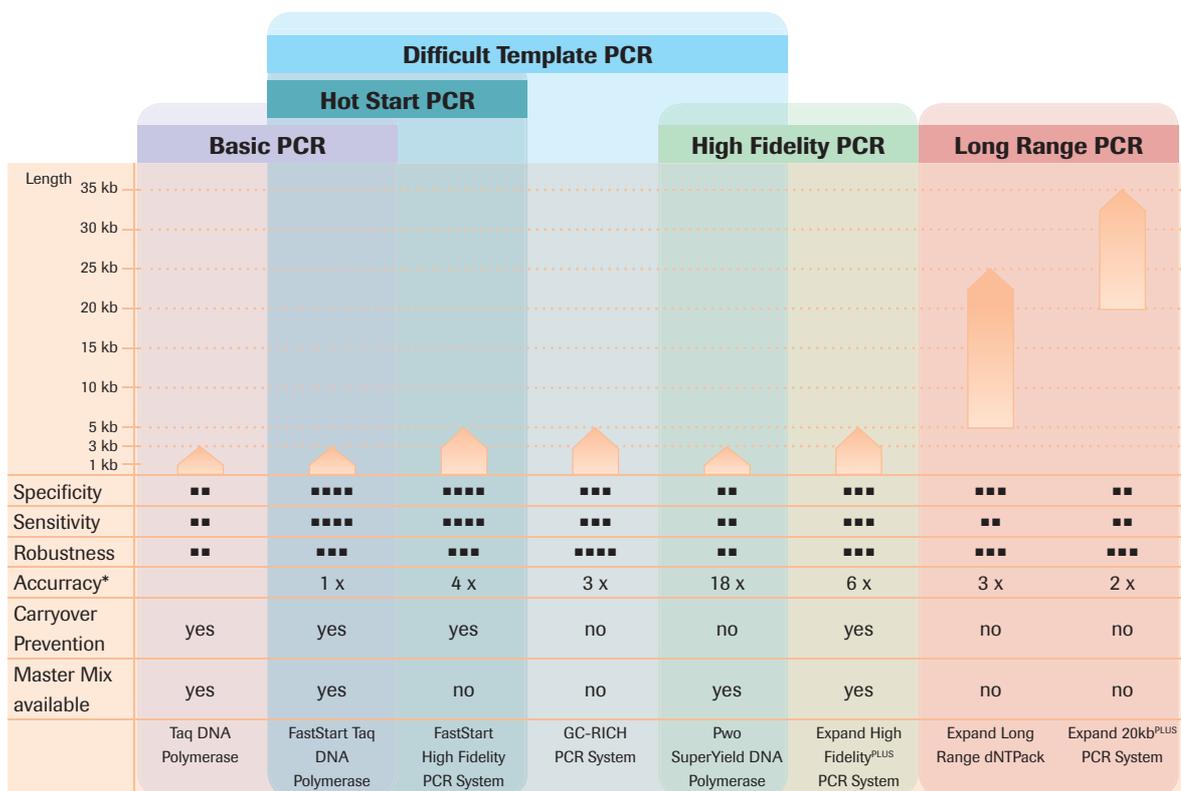
Transcriptor Reverse Transcriptase (available from Roche Applied Science) can be used in two-step RT-PCR to amplify RNA targets up to 14 kb long.

You can choose from several one-step RT-PCR Systems, depending on the requirements of your RT-PCR assay (e.g., length of amplicon, GC content, error rate). The Titan One Tube RT-PCR System is best for amplicons up to 6 kb length but moderate GC content. The *C. therm.* Polymerase (both available from Roche Applied Science) is an efficient enzyme for one-step RT-PCR of RNA targets up to 3 kb long.

Tth DNA Polymerase (available from Roche Applied Science) will reversely transcribe RNA templates (in the presence of  $Mn^{2+}$  ions) and thus may be used for one-step, one-tube RT-PCR of short (up to 1 kb) RNA templates.

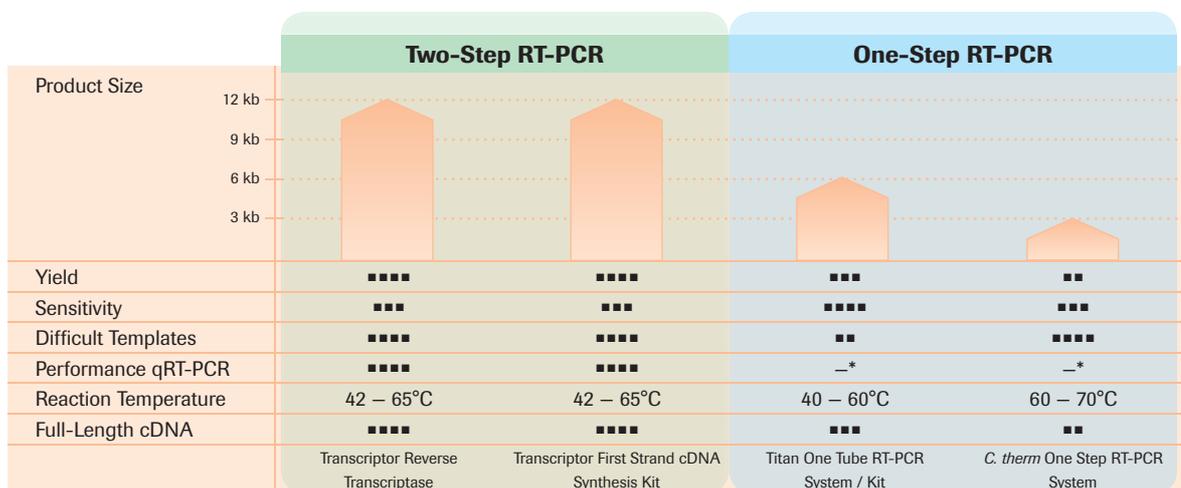
 For a complete listing of RT-PCR enzymes available from Roche Applied Science, see the Ordering Information in the Appendix. For more information on the properties of these enzymes and their appropriateness for specific applications, see “Choosing RT-PCR Enzymes” (in Chapter 5, section 5.1) and the RT-PCR Selection Guide (in the Appendix).

**Table 2.1: Comparison of PCR- and RT-PCR Enzymes.**



\* compared to Taq DNA Polymerase

**Comparison of PCR Enzymes.**



\* for One-Step RT-PC we recommend to use our LightCycler® kits

**Comparison of RT-PCR Enzymes.**

Abbreviations: kb, kilobase pairs  
 Explanation of terms:  
 Processivity: Number of bp that can be added to a copy by one molecule of polymerase before it falls off the template.  
 Length: Maximum PCR target length that can be amplified from a human genomic DNA template with good yield.  
 Specificity: Amplifies only the target of interest.  
 Yield: Produces large amounts of products in a given number of PCR cycles.  
 Reproducibility: Gives the same results from reaction to reaction.  
 Sensitivity: Amplifies template present at a low copy number (fewer than 500 copies)  
 Robustness: Amplifies template even in the presence of contaminating agents or high GC content.  
 Accuracy: Amplifies template without introducing excessive transcriptional errors.

## 2.2.3 Other Important Reaction Components

### 2.2.3.1 Templates and Primers

#### Quality and Amount of DNA Template

Obviously, the purity and quality of the template are critical to the success of the PCR. For details on how pure the template should be and how to get the best quality template, see Chapter 3.

The amount of template in a reaction also strongly influences performance in PCR. For standard PCR, follow these recommendations:

▶ The maximum amount of human genomic DNA should be 200 ng. Use less if possible.

 *Low amounts of genomic template (e.g., <10 ng human genomic DNA) will require specific reaction modifications, such as increases in cycle number, redesign of primers, use of a “Hot Start” reaction, etc.*

▶ For initial experiments, use:

▶ 1–10 ng bacterial DNA

▶ 0.1–1 ng plasmid DNA

▶ 2  $\mu$ l cDNA (as template for a 50  $\mu$ l PCR)

 *When using cDNA as template, do not let the volume of the cDNA exceed 10% of the volume of the PCR mixture (e.g., for a 50  $\mu$ l PCR mixture, use no more than 5  $\mu$ l cDNA from a reverse transcriptase reaction). Greater amounts of cDNA may inhibit the PCR.*

#### Suitable Primers

In most PCR applications, it is the sequence and the concentration of the primers that determine the overall assay success.

For convenience, several primer design software programs are available. These can be used to ensure that the primer sequences are suitable for the reaction.

 *For details on primer design and concentration, see Chapter 3.*

### 2.2.3.2 Nucleotides and Magnesium Ions

#### High Quality Nucleotides

Nucleotides are vital components in amplification reactions and the purity of these reagents significantly influences PCR results. Be aware that not all preparations of nucleotides are acceptable for PCR.

Specifically, many nucleotide preparations contain trace amounts of contaminants (pyrophosphate, mono-, di- and tetraphosphate nucleotides and organic solvents) that can inhibit amplification reactions. Such nucleotide preparations are suboptimal for PCR.

In contrast, Roche Applied Science uses a process to synthesize nucleotides that drastically reduces the amount of contaminants. These high quality (“PCR Grade”) nucleotides are virtually free of all contaminants and are now accepted in the scientific community as the standard nucleotides that should be used for PCR.

Since nucleotides only contribute approx. 1—2% to the total cost of a PCR reaction, buying nucleotides from low cost suppliers will not save very much money. However, considerable costs can be incurred if PCRs are inhibited by impure nucleotides and have to be repeated. For best results, use only PCR Grade nucleotides from Roche Applied Science.

### Concentration of Deoxynucleoside Triphosphate (dNTPs)

During PCR, always use balanced solutions of all four dNTPs to minimize polymerase error rate. Imbalanced (*i.e.*, unequal) concentrations of dNTP will reduce the fidelity of the thermostable DNA polymerase.



*An exception to this rule is the use of dUTP for carryover prevention. A higher concentration of dUTP is usually used in place of dTTP. This is due to differences in the rates at which dTTP and dUTP are incorporated in DNA. For more information, see “Preventing Carryover” in Chapter 4 of this manual.*

The usual concentration of dNTP in standard PCR is 200  $\mu\text{M}$  (each nucleotide). For some applications, concentrations ranging from 50 to 500  $\mu\text{M}$  may be acceptable.



*If you increase the concentration of dNTPs, you must also increase the concentration of  $\text{Mg}^{2+}$  ion in the reaction. Increases in dNTP concentration reduce free  $\text{Mg}^{2+}$ , thus interfering with polymerase activity and decreasing primer annealing.*

### Concentration of Magnesium Ions

Most thermostable DNA polymerases require a source of divalent cations to function. In most cases, the divalent cation required is  $\text{Mg}^{2+}$ .  $\text{Mg}^{2+}$  influences enzyme activity and increases the  $T_m$  of double-stranded DNA.  $\text{Mg}^{2+}$  forms soluble complexes with dNTPs to produce the actual substrate that the polymerase recognizes.

In general, lower  $\text{Mg}^{2+}$  concentrations lead to specific amplification and higher concentrations produce more nonspecific amplification.



*A few DNA polymerases (such as Tth DNA Polymerase) use  $\text{Mn}^{2+}$  rather than  $\text{Mg}^{2+}$ . However, in general, DNA polymerase reactions in the presence of  $\text{Mn}^{2+}$  make DNA copies with significantly lower fidelity than in reactions in the presence of  $\text{Mg}^{2+}$ .*

The concentration of free  $\text{Mg}^{2+}$  depends on the concentrations of compounds that bind the ion, including dNTP, template DNA, primers, free pyrophosphate ( $\text{PP}_i$ ) and EDTA. Therefore, determining the correct  $\text{Mg}^{2+}$  concentration in a PCR is not easy.

The most commonly used concentration of  $\text{Mg}^{2+}$  used in standard PCR with Taq DNA Polymerase is 1.5 mM (with 200  $\mu\text{M}$  dNTPs). However, for best results, always determine the optimal  $\text{Mg}^{2+}$  concentration for each reaction system empirically. Try a range of  $\text{Mg}^{2+}$  concentrations, from 1 mM to 10 mM.



*If you optimize no other reaction component, at least optimize the  $\text{Mg}^{2+}$  concentration. For details on optimizing the  $\text{Mg}^{2+}$  concentration, see “Optimizing the  $\text{Mg}^{2+}$  Concentration for the Standard PCR Protocol” (Chapter 4, section 4.7).*

### 2.2.3.3 Other Reaction Components

#### Appropriate Buffers

Always use only the purest buffers in PCR. These buffers should be designated “PCR Grade.”

In most cases, the reaction buffer supplied with the PCR enzyme(s) will give optimal results with that enzyme. The pH of that buffer will generally be between pH 8.3 and 9.0.



*An easy way to test the effect of varying pH on the PCR is to use the Roche Applied Science PCR Optimization Kit. For details, see Chapter 4, section 4.7.*

#### Reaction Additives and Enhancers

In some cases, adding the following compounds can enhance the efficiency, specificity or yield of standard PCR with Taq DNA Polymerase:

**Table 2.2: Effect of Additives on a Standard PCR.**

Additive/	Recommended Concentration in Reaction <sup>a</sup>	Effect of Additive <sup>a</sup>
Ammonium sulfate [(NH <sub>4</sub> ) <sub>2</sub> SO <sub>4</sub> ] <sup>b</sup>	5 - 30 mM	Facilitates DNA strand separation
Bovine serum albumin *	50 - 500 ng per 50 µl reaction	Binds many PCR inhibitors found in tissue samples
Dimethylsulfoxide (DMSO) <sup>b</sup>	2 - 10% v/v	Lowers <i>T<sub>m</sub></i> of target DNA to enhance annealing
Dimethylformamide (DMF)	<10% v/v <sup>c</sup>	Lowers <i>T<sub>m</sub></i> of target DNA to enhance annealing
Betain		Lowers <i>T<sub>m</sub></i> of target DNA to enhance annealing
Formamide	1.25 - 10% v/v <sup>c</sup>	<ul style="list-style-type: none"> <li>▶ Changes <i>T<sub>m</sub></i> of primer-template hybridization to increase specificity and yield</li> <li>▶ Stabilizes Taq DNA Polymerase</li> </ul>
Gelatin <sup>b</sup>	0.01- 0.10% w/v	Stabilizes Taq DNA Polymerase
Glycerol <sup>b</sup>	5 - 15% v/v	Stabilizes Taq DNA Polymerase
PEG 6000	5 - 15% v/v	Stabilizes Taq DNA Polymerase
SDS	less than 0.01% w/v <sup>c</sup>	Prevents aggregation of polymerase
Spermidine		Reduces nonspecific binding of polymerase to template DNA
T4 Gene 32 protein *	0.05 - 0.1 nmol per 50 µl reaction	Changes <i>T<sub>m</sub></i> of primer-template hybridization to increase specificity and yield
Triton X-100	0.01% v/v	Prevents aggregation of polymerase
Urea	1 - 1.5 M <sup>c</sup>	Lowers <i>T<sub>m</sub></i> of target DNA to enhance annealing

<sup>a)</sup> Part of the information in this table is from Aoyagi (2001). The effects of the additives were determined only for reactions with Taq DNA Polymerase.

<sup>b)</sup> Component of the Roche Applied Science PCR Optimization Kit. For details on optimizing PCR with the kit, see Chapter 4, section 4.7.

<sup>c)</sup> Higher concentrations are inhibitory.

\* Available from Roche Applied Science.

#### Further Reading

For more information on choosing the correct PCR equipment and reagents, see Aoyagi (2001), Cohen (1995), Gelfand (1992a, 1992b), McPherson and Møller (2000b).

## 2.2.4 How Cycling Parameters Affect a PCR

Sections 2.2.2 and 2.2.3 above show how different reaction components affect the PCR. Not surprisingly, the thermal cycling program also greatly affects the chances for a successful PCR. For example:

- ▶ The denaturation time must be long enough to fully denature the template, but short enough not to inactivate the thermostable DNA polymerase.
- ▶ The optimal annealing temperature depends on the melting temperature of the primers.
- ▶ The optimal elongation time depends on the length of the target to be amplified.
- ▶ The optimal number of cycles depends on the abundance of the target in the starting sample (e.g., rarer targets require more cycles to amplify).

Because the optimal cycling parameters vary with each experimental system, the thermal cycling program given in any publication (including the protocols of this manual) should be considered guidelines only. For optimal results, these parameters should be optimized empirically for your particular experimental system and equipment.

The table below summarizes the effects of under- or overshooting the optimal time and temperature for each stage of the thermal cycle. You can use that information to help you determine the optimal PCR parameters for your system.

**Table 2.3: How Cycling Parameters Affect PCR.**

Cycling Parameter	Value Used in Standard PCR <sup>a</sup>	Effect if Parameter Value Is Lower than Optimal <sup>b</sup>	Effect if Parameter Value Is Higher than Optimal <sup>b</sup>
Initial Denaturation	94°C 2 min	Few or no PCR products	Premature denaturation of polymerase, leading to reduced yield
Denaturation during Cycling	94°C 15 – 30 s	Reduced yield	Reduced yield
Primer Annealing	50 to 65°C <sup>c</sup> 30 – 60 s	Reduced yield	Formation of nonspecific products
Elongation	72°C 45 s – 2 min <sup>d</sup>	Reduced yield	▶ Reduced yield ▶ Increased error rate
Total Number of Cycles	25 – 30	Reduced yield	Formation of nonspecific products
Final Elongation	72°C 7 min	Products are not fully double-stranded	Formation of nonspecific products

<sup>a)</sup> With Taq DNA Polymerase; copied from standard PCR protocol (Chapter 4, section 4.1). Other enzyme systems will require different parameter values.

<sup>b)</sup> From Aoyagi (2001). Effects are generally independent of the enzyme system used.

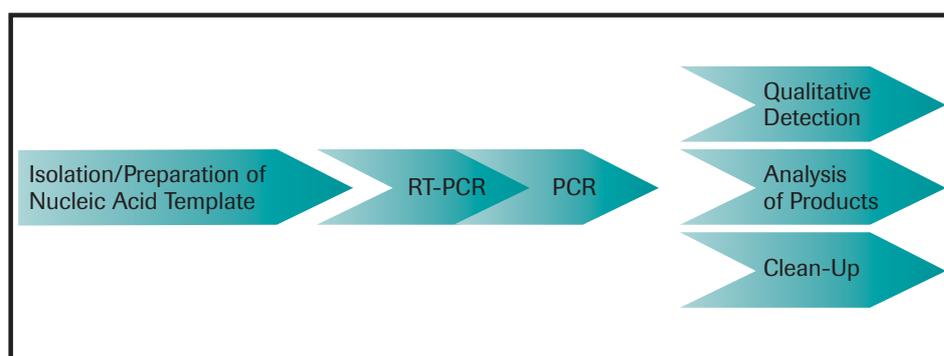
<sup>c)</sup> Optimal temperature depends on primer melting temperature.

<sup>d)</sup> Elongation time depends on fragment length; for Taq DNA Polymerase, the recommended times are 45 seconds for targets that are 1 kb or shorter, 1 minute for 1.0 – 1.5 kb, and 2 minutes for 1.5 – 3.0 kb.

## 2.3 Typical Workflow for PCR/RT-PCR

Now that you have learned how to prevent contamination in your laboratory (section 2.1) and set up a PCR (section 2.2), you are ready to apply the power of PCR to your research.

Even though there are many different ways to use PCR, the overall workflow for almost all PCR and RT-PCR experiments involves the same general steps:



You can think of this workflow as links in a chain. As everyone knows, a chain is only as strong as its weakest link. Therefore, Roche Applied Science designs and tests its reagents, kits, instruments and experimental protocols as elements of a chain. By using the Roche Applied Science products and protocols described in this manual, you can rest assured that:

- ▶ The information in this manual will help you choose the right products and protocols for your specific application.



*For a quick overview of how the information in this manual can help you choose the right products and protocols to forge the strongest “PCR workflow chain,” see the table below.*

- ▶ Each product and protocol is optimized for its designated applications.
- ▶ All elements of the Roche Applied Science product/protocol chain will work smoothly together.
- ▶ Using Roche Applied Science products and protocols will reduce the complexity of your PCR experiments, saving you both time and money.



*There are sophisticated, real-time PCR instruments available (e.g., the LightCycler® 1.5 Instrument, the LightCycler® 2.0 Instrument and the LightCycler® 480 System, all from Roche Applied Science) that perform some of the workflow steps concurrently. (If you want a brief look at the power of these real-time PCR instruments, see Chapter 7.)*

**Table 2.4: Overview of How This Manual Can Help You Choose the Right Products and Protocols.**

Isolation of Nucleic Acids	Amplification of Nucleic Acids	Qualitative Detection	Clean-up and Analysis (optional)
<p>Manual or automated isolation of RNA (See Chapter 3)</p> <ul style="list-style-type: none"> <li>▶ Total RNA</li> <li>▶ Messenger RNA</li> <li>▶ Viral RNA</li> </ul>	<p style="text-align: center;"><b>Reverse Transcription and Amplification of RNA Templates</b></p> <p>Before you start:</p> <ul style="list-style-type: none"> <li>▶ Prevent degradation of RNA (See section 5.2 in Chapter 5)</li> <li>▶ Choose the correct enzymes, kits, etc. (See section 5.1 in Chapter 5 and the RT-PCR Product Selection Guide in the Appendix)</li> </ul> <p>Via: separate reverse transcription and amplification steps (Two Step RT-PCR; see section 5.4 in Chapter 5) or coupled reverse transcription and amplification (One Step RT-PCR; see section 5.3 in Chapter 5)</p> <p>Applications:</p> <ul style="list-style-type: none"> <li>▶ RT-PCR of normal templates (See sections 5.3 and 5.4 in Chapter 5)</li> <li>▶ RT-PCR of GC rich templates (See sections 5.3 and 5.4 in Chapter 5)</li> <li>▶ RT-PCR of long templates (See section 5.4 in Chapter 5)</li> <li>▶ Special RT-PCR applications (See Chapter 8)</li> </ul>	<p>Agarose gel electrophoresis (See Chapter 6)</p>	<p>Post PCR purification (See Chapter 6)</p> <p>Cloning of PCR products (See Chapter 6)</p>
<p>Manual or automated isolation of DNA (See Chapter 3)</p> <ul style="list-style-type: none"> <li>▶ Genomic DNA</li> <li>▶ Plasmid DNA</li> <li>▶ Viral DNA</li> </ul>	<p style="text-align: center;"><b>Amplification of DNA Templates</b></p> <p>Before you start: Choose the correct enzymes, nucleotides, kits, etc. (See section 4.6 in Chapter 4 and the PCR Product Selection Guide in the Appendix)</p> <p>Applications:</p> <ul style="list-style-type: none"> <li>▶ Overview: PCR Protocol Selection Guide (See Chapter 4)</li> <li>▶ Amplification of normal templates (See sections 4.1 through 4.2 in Chapter 4)</li> <li>▶ Amplification of long templates (See section 4.3 in Chapter 4)</li> <li>▶ Amplification of difficult templates (See section 4.4 in Chapter 4)</li> <li>▶ Prevention of carryover contamination (See section 4.5 in Chapter 4)</li> <li>▶ Multiplex reactions (See Chapter 8)</li> <li>▶ High throughput analysis (See Chapter 7)</li> <li>▶ Special PCR applications (See Chapter 8)</li> </ul>		
	<p>Quantitative, real-time PCR Analysis (See Chapter 7)</p>		

**Disclaimer**

For Titan One Tube RT-PCR System, Cat. Nos. 11 888 382 001, 11 855 476 001; Titan One Tube RT-PCR Kit, Cat. No. 11 939 823 001; *C.therm.* Polymerase One-Step RT-PCR System, Cat. Nos. 12 016 338 001, 12 016 346 001; Tth DNA Polymerase, Cat. Nos. 11 480 014 001, 11 480 022 001, and Protector RNase Inhibitor, Cat. Nos. 11 480 014 001, 11 480 022 001 see Disclaimer No. 1.

For Pwo DNA Polymerase, Cat. Nos. 11 644 947 001, 22 644 955 001; Pwo SuperYield DNA Polymerase, Cat. Nos. 04 340 868 001, 04 340 850 001, Pwo SuperYield DNA Polymerase, dNTPack, Cat. Nos. 04 743 750 001, 04 743 776 001, and Pwo Master, Cat. No. 03 789 403 001, see Disclaimer No. 2.

For the PCR Master, Cat. No. 11 636 103 001 see Disclaimer No. 3.

For the PCR Core Kit <sup>PLUS</sup>, Cat. No. 11 578 553 001 see Disclaimer No. 4.

*Chapter 3*  
**Primer and Template  
Preparation**

3

<b>3</b>	<b>Primer Design and Template Preparation</b>	<b>Page</b>
3.1	Primer Design .....	41
3.1.1	Strategies for Designing Good Primers.....	41
3.1.2	Design of PCR Primers: Helpful Web Sites that Contain Tips, Software, and Sequences .....	42
3.1.3	Using the Primers in PCRs.....	43
3.2	Template Preparation .....	45
3.2.1	Overview.....	45
3.2.2	Products for Manual and Automated Isolation of Nucleic Acids.....	48
3.3	Protocols for Isolation of Typical Templates.....	54
3.3.1	Isolation of High Molecular Weight Nucleic Acids from Cultured Cells with the HIGH PURE PCR Template Preparation Kit .....	54
3.3.2	Isolation of Total RNA from Whole Blood with the HIGH PURE RNA Isolation Kit .....	57
3.3.3	Isolation of cDNA with the HIGH PURE PCR Product Purification Kit .....	60

# 3

## 3. Primer Design and Template Preparation

### 3.1 Primer Design and Use

In most PCR applications, it is the sequence and the concentration of the primers that determine the overall assay success. This section presents a brief overview of how to determine and optimize these parameters.

#### 3.1.1 Strategies for Designing Good Primers

Before designing primers, consider the following points:

- ▶ What are the goals of your PCR? Do you want to e.g. amplify a few or many targets, clone the full-length coding region, quantify the target, or identify unknown sequences?
- ▶ What are the potential problems you need to consider? For example, could pseudogenes cause problems? If you are analyzing SNPs (single nucleotide polymorphisms), what are the most likely effects of mutant sequences? (See, for example, Kwok et al., 1995.)

Once you have identified what the PCR needs to accomplish and to avoid, follow the rules for good primer design. Several software programs (see Table 3.1.2 below) are available for sequence analysis and primer design. These can be used to ensure that the primer sequences have the following general characteristics:

- ▶ Are 18–30 bases long (if the PCR enzyme is Taq DNA Polymerase; this number may change for enzymes with greater heat stability). Longer primers give more specificity but tend to anneal with lower efficiency, leading to decreased yield.
- ▶ Recognize the target sequence specifically. Shorter primers (less than 15 nucleotides long) anneal very efficiently but they may not be specific enough. Longer primers may be useful to distinguish genes with a high degree of sequence homology.



*The probability of finding a match to a set of primers with 20 nucleotides is  $(\frac{1}{4})^{(20+20)} = 9 \times 10^{-26}$  (Cha and Thilly, 1995).*



*For RT-PCR, primers should span exon-exon junctions to avoid amplifying genomic DNA (Huang, Fasco and Kaminsky, 1996).*

- ▶ Contain no internal secondary structure.
- ▶ Have G/C content between 40% and 60%.
- ▶ Have a balanced distribution of G/C and A/T rich domains.
- ▶ Are not complementary to each other at their 3' ends and are not self complementary.



*Primer-dimers will form if the primers have one or more complementary bases so that base pairing between the 3' ends of the two primers can occur.*

# 3

- ▶ Have a melting temperature ( $T_m$ ) that allows annealing to occur between 55° and 65°C.



*Primers in a set should have similar melting temperatures. For maximum specificity, keep melting temperature between 62°C and 65°C.*

*Remember that optimal primer-template annealing temperatures are often approx. 5 to 10°C higher than the  $T_m$  of the primers.*

*Bases that do not hybridize to the template may be added to the 5' end of a primer (e.g., to introduce restriction sites into the amplification product).*



*For special recommendations when designing primers for Long Range PCR please refer to Chapter 4, 4.3 Long Template PCR, page 92.*

# 3

## 3.1.2 Design of PCR Primers: Helpful Web Sites that Contain Tips, Software, and Sequences

Here, we outline some ways to get more information on the Web about:

- ▶ Basics of primer design
- ▶ Software for designing PCR primers

### Basics of Primer Design

The best way to learn how to design primers is to ask someone who has already had success in designing primers. Fortunately, the Web makes it possible to find and query such people from all over the world. Table 3.1.1 is a sampling of Web sites that provide tutorials, lectures, papers, and tips on primer design.



*We found these sites in a routine search of the Web in January, 2006. We have not evaluated or verified the information contained in them. The URL address as well as the content may change or even disappear without notice.*

**Table 3.1.1: Sources of information on designing PCR primers.**

For information on	Consult this URL (Web address)
Basic PCR Information:	
The PCR Jump Station	<a href="http://www.highveld.com/pages/pcr.html">http://www.highveld.com/pages/pcr.html</a>
Links to many sources of basic PCR: ExPASy	<a href="http://www.expasy.ch">http://www.expasy.ch</a>
NCBI: National Center for Biotechnology Information	<a href="http://www.ncbi.nlm.nih.gov/Genbank/index.html">http://www.ncbi.nlm.nih.gov/Genbank/index.html</a>
Primer design software	<a href="http://www.ebi.ac.uk/biocat/biocat.html">http://www.ebi.ac.uk/biocat/biocat.html</a> <a href="http://www.highveld.com/pages/pcr-primer-design.html">http://www.highveld.com/pages/pcr-primer-design.html</a> <a href="http://www.pcrlinks.com/generalities/primers.htm">http://www.pcrlinks.com/generalities/primers.htm</a>
General hints on primer design	<a href="http://www.protocol-online.org/prot/Molecular_Biology/PCR/">http://www.protocol-online.org/prot/Molecular_Biology/PCR/</a>
Gene search and validation BLAST	<a href="http://www.ncbi.nlm.nih.gov/blast/producttable.shtml">http://www.ncbi.nlm.nih.gov/blast/producttable.shtml</a>

## Software for Designing PCR Primers

Computer programs simplify the complex task of designing PCR primers. In addition to the many commercial primer design programs available, there are an ever increasing number of free programs available to anyone with an on-line connection. Table 3.1.2 contains examples of software titles we found by searching the Web for “primer design.”



*We have not evaluated the programs listed in this table. For more information on these programs and an updated list of primer design programs available, see the Bio-Catalog page at <http://www.ebi.ac.uk/biocat/biocat.html>.*

**Table 3.1.2: Primer Design Programs Available on the Web**

Software	Operating systems	Available from <sup>a</sup>
CODEHOP	Online	<a href="http://blocks.fhcrc.org/codehop.html">http://blocks.fhcrc.org/codehop.html</a>
FastPCR	Microsoft Windows	<a href="http://www.biocenter.helsinki.fi/bi/Programs/fastpcr.htm">http://www.biocenter.helsinki.fi/bi/Programs/fastpcr.htm</a>
GeneFisher Interactive Primer Design	Online	<a href="http://bibiserv.techfak.uni-bielefeld.de/genefisher/">http://bibiserv.techfak.uni-bielefeld.de/genefisher/</a>
Gene search and validation BLAST	Online	<a href="http://www.ncbi.nlm.nih.gov/blast/producttable.shtml">http://www.ncbi.nlm.nih.gov/blast/producttable.shtml</a>
Primer3 (Pick primers from a DNA sequence)	Online	<a href="http://frodo.wi.mit.edu/cgi-bin/primer3/primer3_www.cgi">http://frodo.wi.mit.edu/cgi-bin/primer3/primer3_www.cgi</a>
PCR Suite (a collection of programs that interact with the Whitehead's Primer3 program.)	Online	<a href="http://www2.eur.nl/fgg/kgen/primer/index.html">http://www2.eur.nl/fgg/kgen/primer/index.html</a>
ProbeFinder Software (which uses Primer3 for primer design)	Online	<a href="http://www.universalprobelibrary.com">http://www.universalprobelibrary.com</a>  <i>For more information on the ProbeFinder Software and the Universal Probe Library, see Chapter 7.</i>
PrimerX	Online	<a href="http://bioinformatics.org/primerx/">http://bioinformatics.org/primerx/</a>
Primo Pro	Online	<a href="http://www.changbioscience.com/primo/primo.html">http://www.changbioscience.com/primo/primo.html</a>

<sup>a)</sup> List compiled in January, 2006. Subject to change and update.

### 3.1.3 Using the Primers in PCRs

The information below applies to PCRs performed with FastStart Taq DNA Polymerase and Taq DNA Polymerase. Other PCR enzymes may require different conditions. See Chapter 4 for more information.



*When testing new primers in a PCR, always include a positive control reaction with a template that has been tested for function in PCR. This control shows whether the primers are working. For example, Human Genomic DNA (from Roche Applied Science) provides a good control template for evaluation of human primer sequences.*

### Primer Concentration

Primer concentrations between 100 and 900 nM are generally optimal. On the other hand, for some systems, a higher primer concentration (up to 1  $\mu$ M) may improve results.

Higher primer concentrations may promote mispriming and accumulation of non-specific product. Lower primer concentrations may be exhausted before the reaction is completed, resulting in lower yields of desired product.



*When the target is rare or the template amount is low, validation of primer concentration is especially important. In this case, less primer is needed since too much primer will cause primer-dimers.*

### Choosing the Primer Annealing Temperature

The choice of the primer annealing temperature is probably the most critical factor in designing a high specificity PCR. If the temperature is too high, no annealing occurs; if it is too low, non-specific annealing will increase dramatically.

Therefore, in most cases, annealing temperature must be determined and optimized empirically. Optimal annealing temperatures are often approx. 5° to 10°C higher than the  $T_m$  of the primers



*For both primers, the  $T_m$  (and hence the annealing temperature) should be similar.*

### Choosing Primer Extension Conditions

For fragments up to 3 kb, primer extension is normally done at 72°C. Taq DNA Polymerase can add approximately 60 bases per second at 72°C.

A 45-second extension is sufficient for fragments up to 1 kb. For extension of fragments up to 3 kb, allow about 45 seconds per kb. However, these times may need to be adjusted for specific templates.



*For improved yield, use the cycle extension feature of the thermal cycler. For instance, perform the first 10 cycles at a constant extension time (e.g., 45 s for a 1 kb product). Then, for the next 20 cycles, increase the extension time by 2 – 5 s per cycle (e.g. 50 s for cycle 11, 55 s for cycle 12, etc.). Cycle extension allows the enzyme more time to do its job, because as PCR progresses, there is more template to amplify and, since enzyme is denatured during the prolonged high PCR temperatures, less enzyme to do the extension.*

## 3.2 Template Preparation

### 3.2.1 Overview

The key to successful PCR is the purity of the nucleic acid template used. The main purpose of template purification, in addition to concentrating the target sequence, is to remove PCR inhibitors from the preparation. Many PCRs fail due to impure nucleic acids or templates containing contaminants that negatively influence PCR performance.

To get the purest, inhibitor-free templates, always use a purification product specifically designed to purify DNA or RNA from a specific source (such as the reagents and kits described in this chapter). These are specifically designed to remove nucleases and PCR inhibitors that otherwise might co-purify with the target.

#### Requirements for PCR Templates

A good DNA template preparation should:

- ▶ Have a high degree of integrity. That is, it should be of high molecular weight, undegraded and unnicked.



*After isolation, intact sample DNA may still degrade if:*

- ▶ *Nuclease has not been completely removed during the purification process.*
- ▶ *The sample becomes contaminated with nucleases during repeated use. Always aliquot the sample into smaller portions that will be used only in a single experiment.*



*DNA isolated from paraffin-embedded tissue sections or archived, fixed tissues may have been extensively nicked during tissue preparation (Aoyagi, 2001). Each human cell contains about 2 meters of DNA, so a typical method for isolating DNA from tissue will shear that DNA.*



*To check the size and quality of the DNA, run an aliquot on an agarose gel.*

- ▶ Free of inhibitors. Impure templates may contain polymerase inhibitors such as porphyrins, humic acid, heparin, sodium citrate, phenol, chloroform, EDTA, SDS, xylene, cyanol and some heavy metals that decrease the efficiency of the reaction.
  - ▶ Pure. It should have an  $A_{260} : A_{280}$  ratio of 1.8 to 2.0, indicating that it is free of protein and carbohydrate.
  - ▶ Free of RNA. Large amounts of RNA in a DNA template can chelate  $Mg^{2+}$  and reduce the yield of the PCR.
- ▶ *Analogously, if RNA is the template (e.g., for one-step RT-PCR), it should be free of DNA.*

## Template Amount

The amount of template in a reaction strongly influences performance in PCR. For standard PCR with Taq DNA Polymerase, follow these recommendations:

- ▶ Human genomic DNA: The maximum amount should be 500 ng. Use less if possible.



*Low amounts of genomic template (e.g., <10 ng human genomic DNA) will require specific reaction modifications, such as increases in cycle number, redesign of primers, use of a “Hot Start” reaction, etc.*

- ▶ Bacterial DNA: 1 – 10 ng.

- ▶ Plasmid DNA: 0.1 – 1.0 ng.

- ▶ cDNA: 2 µl (as template for a 50 µl PCR).



*When using cDNA as template, do not let the volume of the cDNA exceed 10% of the volume of the PCR mixture. (e.g., for a 50 µl PCR mixture, use no more than 5 µl cDNA from a reverse transcriptase reaction). Greater amounts of cDNA may inhibit the PCR.*

*When testing a new template, always include a positive control with primers that amplify a product of known size and produce a good yield.*

An example of a good positive control might be a sample that contains a high copy number of the target sequence. A good endogenous positive control would be a gene target that is constitutively expressed in the same sample as the target; the expression levels of the control should change only minimally even if the expression of the target varies greatly.

*Example:* If the target is human genomic DNA, our Human tPA (Tissue Plasminogen Activator) Control Primer Set (for amplification of 4.8 kb, 9.3 kb, and 15.0 kb targets) can serve as a positive control.

It is also important to include a negative control in a PCR run. For example, a suitable negative control might be:

- ▶ A sample that contains the target, but is amplified with only one primer,
- ▶ A reaction that contains no Mg<sup>2+</sup> ion,
- ▶ A reaction that contains no enzyme,
- ▶ A sample known to lack the target sequence, or
- ▶ A “cDNA” from an RT step performed without reverse transcriptase.

## Guidelines for Isolation of DNA and RNA Templates

### DNA Isolation

Key factors for a successful DNA isolation include:

- ▶ If possible start with a fresh sample.
- ▶ Process your sample as quickly as possible.
- ▶ Carefully select the homogenization method according to the characteristics of your sample.
- ▶ Do not use too much starting material. Too much starting sample might cause an increase in the viscosity of the DNA preparation and lead to shearing of genomic DNA.
- ▶ Always be aware of nucleases. For example, to prevent degradation, make sure that the sample is lysed quickly and completely, and that the lysis buffer has inactivated nucleases.
- ▶ To ensure that your DNA is not sheared during the isolation process, avoid extensive vortexing or repeated pipetting.
- ▶ Make sure the isolated sample is free of chemical contaminants (*e.g.*, ethanol, phenol, SDS, guanidinium isothiocyanate) used in the isolation process, since they will interfere with downstream applications, especially PCR.
- ▶ After DNA isolation: Repeated use of isolated sample DNA can lead to nuclease degradation. This may be due to incomplete removal of nucleases during sample preparation or contamination during repeated use of the same sample preparation. Avoid repeated freeze-thaw cycles as this may lead to shearing of complex DNA. Complex genomic DNA is best stored at +2 to 8°C.

### RNA Isolation

For RT-PCR, you can either use total RNA or mRNA. Though total RNA is routinely used as the template for RT-PCR, some rare messages (or downstream cloning applications) require use of mRNA.

Key factors for successful RNA isolation include:

- ▶ Avoid all RNase contamination. For example, always work under sterile, RNase-free conditions. It is extremely important to use only RNase-free glassware, plasticware and solutions for all procedures involved in RNA isolation and storage.
- ▶ If possible, start with a fresh sample.
- ▶ Process your sample as quickly as possible.
- ▶ Carefully select the homogenization method according to the characteristics of your sample.
- ▶ Make sure the isolated sample is free of chemical contaminants (*e.g.*, ethanol, phenol, SDS, guanidinium isothiocyanate) used in the isolation process, since they will interfere with downstream applications, especially PCR.
- ▶ Make sure that your RNA samples are stored below pH 8.0 and at low temperatures of -50 to -80°C. Take special care to prevent introducing RNase contamination into the stored sample.

# 3

### 3.2.2 Products for Manual and Automated Isolation of Nucleic Acids

Since a plethora of methods exists for extraction and purification of nucleic acids, researchers usually choose the technique most suited to their:

- ▶ Target nucleic acid (ssDNA, dsDNA, total RNA, mRNA, etc.)
- ▶ Source organism (mammalian, lower eukaryotes, plants, prokaryotes, viruses, etc.)
- ▶ Starting material (whole organ, tissue, cell culture, blood, etc.)
- ▶ Desired results (yield, purity, purification time required, etc.)
- ▶ Downstream application (PCR, RT-PCR, cloning, labeling, blotting, cDNA synthesis, RNase protection assays, etc.)

#### Products from Roche Applied Science for Manual Template Preparation

Roche Applied Science has a wide array of manual nucleic acid preparation products that are specifically designed to prepare DNA or RNA templates from a particular source or for certain applications. See Table 3.2.1 for a list of these products.

Our nucleic acid purification products combine proven, reliable purification methods with innovations that allow you to:

- ▶ Process more samples in less time
- ▶ Minimize nucleic acid loss and degradation
- ▶ Increase laboratory efficiency and safety
- ▶ Avoid organic solvents and toxic reagents

We use four distinct approaches to purify PCR templates and other nucleic acids:

#### 1. Silica adsorption

These kits rely on the tendency of nucleic acids to adsorb to silica (glass) in the presence of guanidine hydrochloride (Melzak, 1996; Vogelstein and Gillespie, 1979). By varying conditions such as the ionic strength and the pH of the surrounding solution, each kit has been optimized to prepare a particular type of nucleic acid from biological samples.



*Some of our purification products based on silica adsorption (e.g., the HIGH PURE Kits) are especially suitable for preparation of PCR or RT-PCR templates; these are described in more detail below. Others are designed to purify PCR products for downstream procedures; these are described in Chapter 6.*

#### 2. Affinity purification

These kits exploit the hybridization properties of nucleic acids to purify particular classes of DNA or RNA.

#### 3. Solution-based isolation

These products use proprietary cell lysis and extraction methods to isolate particular templates.

## 4. Gel filtration

The Quick Spin and mini Quick Spin columns separate molecules based on their relative size. Thus, they can separate DNA fragments from small contaminants (salts, unincorporated nucleotides, primers).



A number of these products can also prepare nucleic acids for other common molecular biology procedures besides PCR. For a complete listing of these nucleic acid preparation products, visit the nucleic acid purification special interest site at [www.roche-applied-science.com/napure](http://www.roche-applied-science.com/napure).

### Product Selection Guide: Products for Template Preparation

Table 3.2.1 lists those products from Roche Applied Science that are most commonly used for template preparation. This table can help you select a product according to the type of PCR template you wish to purify, followed by the favoured source of the nucleic acid and the scale of the purification.

**Table 3.2.1: Roche Applied Science Products Commonly Used for Template Preparation.**

Nucleic Acid		Origin/Source	Scale (Sample Size per Isolation)	Recommended Product <sup>a</sup>
Type	Subtype			
DNA	Genomic	Tissue, cultured cells, bacteria, yeast, blood	<ul style="list-style-type: none"> <li>▶ Tissue: 25-50 mg</li> <li>▶ Cultured cells: 10<sup>4</sup>-10<sup>6</sup></li> <li>▶ Yeast: 10<sup>8</sup></li> <li>▶ Whole blood: 200-300 µl</li> </ul>	HIGH PURE PCR Template Preparation Kit
	Genomic	Tissue, cultured cells, bacteria, yeast, mouse tail	<ul style="list-style-type: none"> <li>▶ Tissue: 100-1000 mg</li> <li>▶ Cultured cells: 1-5 × 10<sup>7</sup></li> <li>▶ Yeast: up to 3 × 10<sup>10</sup></li> <li>▶ Mouse tail: 50-400 mg</li> </ul>	DNA Isolation Kit for Cells and Tissues
	Genomic	Human blood	200-300 µl	HIGH PURE PCR Template Preparation Kit
	Genomic	Mammalian/human blood	10 ml	DNA Isolation Kit for Mammalian Blood
	Genomic	Whole blood/cultured cells	<ul style="list-style-type: none"> <li>▶ Whole blood: 200 µl</li> <li>▶ Cultured cells: 2 × 10<sup>6</sup></li> </ul>	Apoptotic DNA Ladder Kit
	Viral	Serum, plasma, blood, other body fluids, supernatant from cell cultures	200-600 µl	HIGH PURE Viral Nucleic Acid Kit
Viral	Serum, plasma, supernatant from cell cultures	200 µl	HIGH PURE 16 System Viral Nucleic Acid Kit	
DNA fragments	ss cDNA, ds cDNA, PCR products, cRNA	<ul style="list-style-type: none"> <li>▶ ss cDNA: 50 µg</li> <li>▶ ds cDNA: 10 µg</li> <li>▶ cRNA: 150 µg</li> </ul>	Microarray Target Purification Kit	
RNA	mRNA	Cultured cells, tissues, total RNA	<ul style="list-style-type: none"> <li>▶ Cultured cells: up to 5 × 10<sup>5</sup></li> <li>▶ Tissues: up to 20 mg</li> <li>▶ Total RNA: up to 40 µg</li> </ul>	mRNA Capture Kit
	mRNA	Cultured cells, tissues, total RNA	<ul style="list-style-type: none"> <li>▶ Cultured cells: 2 × 10<sup>5</sup> - 10<sup>8</sup></li> <li>▶ Tissues: 50-1000 mg</li> <li>▶ Total RNA: 250-2500 µg</li> </ul>	mRNA Isolation Kit
	mRNA	Whole blood, bone marrow aspirate	1.5 - 5 ml	mRNA Isolation Kit for Blood/Bone Marrow

Nucleic Acid		Origin/Source	Scale (Sample Size per Isolation)	Recommended Product <sup>a</sup>
Type	Subtype			
Total RNA		Cultured cells, bacteria, yeast, blood	<ul style="list-style-type: none"> <li>▶ Cultured cells: 10<sup>6</sup></li> <li>▶ Yeast: 10<sup>8</sup></li> <li>▶ Bacteria: 10<sup>9</sup></li> <li>▶ Blood: 200-500 µl</li> </ul>	HIGH PURE RNA Isolation Kit
Total RNA		Solid tissue	1-10 mg	HIGH PURE RNA Tissue Kit
Total RNA		Cultured cells, tissues, bacteria, yeast, blood, plant cells	<ul style="list-style-type: none"> <li>▶ Tissue: 50-100 mg/ml TriPure</li> <li>▶ Animal, plant or yeast cells: 5-10 × 10<sup>6</sup>/ml TriPure</li> <li>▶ Bacteria: 10<sup>7</sup> cells/ml TriPure</li> </ul>	TriPure Isolation Reagent
Total RNA		Formalin-fixed, paraffin-embedded tissue; fresh-frozen tissue samples	<ul style="list-style-type: none"> <li>▶ Fixed, embedded tissue: 5-10 µg sections</li> <li>▶ Frozen tissue: 10-30 mg</li> </ul>	HIGH PURE RNA Paraffin Kit
Viral RNA		Serum, plasma, other body fluids, supernatant from cell cultures	200-600 µl	HIGH PURE Viral RNA Kit

<sup>a)</sup> For details on the pack sizes of these products, see the Ordering Information in the Appendix.

### The HIGH PURE Kits for PCR Template Preparation

The principle behind the HIGH PURE kits (Figure 3.2.1) is the binding of nucleic acids to silica glass in the presence of chaotropic salts. Depending on the ionic strength and pH of the solution, different types of nucleic acids have different binding characteristics. In each HIGH PURE Kit, this binding method is carefully optimized to isolate a particular type of nucleic acid.

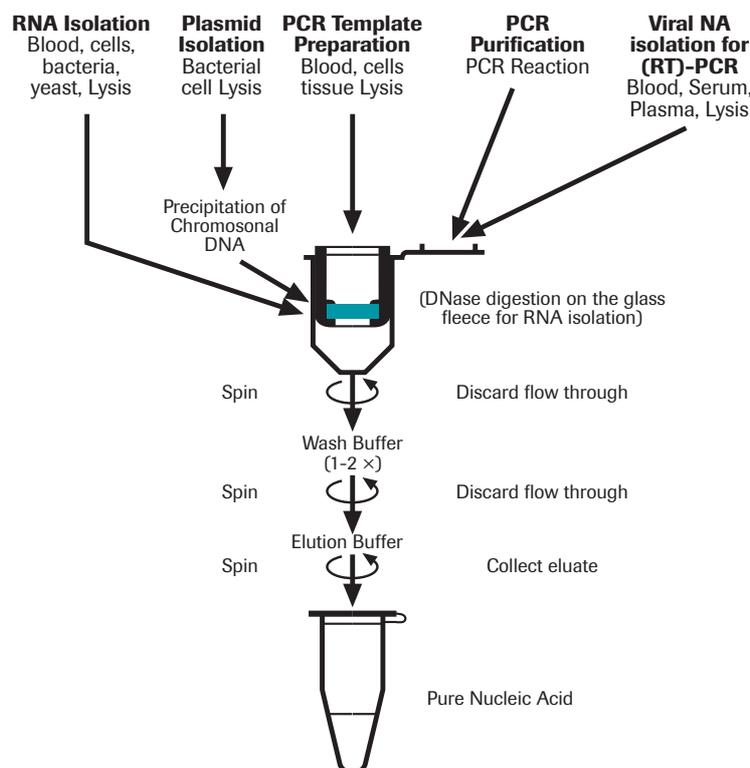


Figure 3.2.1. Principle of HIGH PURE technology.

Each “HIGH PURE” column contains a special glass fiber fleece immobilized in a cylinder that can be centrifuged, allowing fast, efficient washing and elution steps (Figure 3.2.1). For example, the HIGH PURE PCR Template Preparation Kit can isolate genomic DNA from cultured mammalian cells in approximately 20 minutes, requiring a total hands-on time of less than 12 minutes. (See section 3.3.1 for details.) The isolated DNA can be used directly in a PCR. This kit also allows easy, simultaneous processing of multiple samples.

The HIGH PURE product line offers:

- ▶ A wide range of kits, which are designed for the isolation of many types of nucleic acids from different sample materials.
- ▶ Highly purified nucleic acids of high integrity, suitable for amplification.
- ▶ Reliable, reproducible performance.
  - ▶  Roche Applied Science uses ISO-certified operating and testing procedures for the HIGH PURE product line, thus ensuring that the performance of each lot of each product is similar to the next lot.
  - ▶  For detailed information on our product line for manual isolation of nucleic acids, visit [www.roche-applied-science.com/napure](http://www.roche-applied-science.com/napure) or ask your local representative for the following literature:
    - ▶ Nucleic Acid Isolation and Purification Selection Guide
    - ▶ Nucleic Acid Isolation and Purification Applications Manual (2nd edition)

### Products from Roche Applied Science for Automated Sample Preparation



**Figure 3.2.2. MagNA Pure System Instruments available from Roche Applied Science:**  
The MagNA Pure LC Instrument, the MagNA Pure Compact System, and the MagNA Lyser Instrument

### MagNA Pure LC System

The MagNA Pure LC Instrument allows rapid preparation of nucleic acids that are free of cross-contamination. Nucleic acids (*e.g.*, cellular, viral, bacterial, or fungal DNA, RNA, or mRNA) from a broad variety of samples (*e.g.*, blood, blood cells, cultured cells, plasma, serum, sputum, stool, plant tissues or food products) can be purified in approximately one to three hours.

A completely enclosed housing unit, automatic detection of clots and tip loss, and sample tracking capabilities make the MagNA Pure LC Instrument a true "walk-away" instrument for general laboratory use. Filtration, centrifugation, and other manual steps are completely eliminated. Yet, automated purification on the instrument is more reproducible and less subject to cross-contamination than standard manual methods.

The following features make the MagNA Pure LC Instrument an excellent automated instrument for nucleic acid isolation:

- ▶ **Prevention of contamination.** The instrument uses a controlled, piston-driven 8-nozzle pipetting head and positive displacement, without vacuum pumps or tubing, to deliver samples. Therefore, the risk of cross-contamination is reduced to an absolute minimum.
- ▶ **Easy sample and run tracking.** Later versions of the MagNA Pure software (3.0 and above) support a bar-code reader and bar-code printer for easy sample tracking. Sample ID and information can either be entered manually or via a bar-code reader into a table in the software. The software also supports a pager so the user can be notified of the status of the instrument and the isolation process.
- ▶ **Easy run set-up.** After users enter the name of the correct MagNA Pure LC Kit, the appropriate purification protocol for the sample material and the nucleic acid to be purified, they simply specify the number of isolations. Then, the software automatically calculates the sample and elution volumes, the amount of each reagent and the number of reaction tips required for the run. The user places all required reagents in nuclease-free, disposable reagent tubs, and loads samples into sample cartridges that are set into place on the instrument.
- ▶ **Flexibility.** Variable numbers of samples (up to 32) can be processed in one run.
- ▶ **True automation.** All isolation steps are performed automatically. The specially designed nuclease-free disposable reaction tips are used for pipetting the reagents and as reaction vials for the magnetic-bead-based separation of nucleic acids. Isolated nucleic acids are washed in the tips, then eluted from the magnetic glass particles into the cooled sample cartridge.
- ▶ **Safe handling of disposables and waste.** Used reaction tips are automatically discarded into an attached, autoclavable waste bag and liquid waste is pipetted at the end of the run into an appropriate waste tank. After a run, the inside of the housing unit can easily be cleaned with commonly used disinfectants and decontaminated with a built-in UV-lamp. UV decontamination and the HEPA filtration system ensure maximum safety for both samples and environment.

The MagNA Pure LC Instrument can also set up reaction mixtures for downstream PCR / RT-PCR. The post-elution part of the MagNA Pure LC instrument is flexible enough to prepare the nucleic acids for a variety of different PCR formats. Isolated nucleic acids and master mixes can be automatically pipetted into LightCycler® Capillaries, 96-well PCR plates, A-rings, or tubes.

### The MagNA Pure Compact System

The MagNA Pure Compact System offers an automated, benchtop solution for nucleic acid purification. Its small footprint, easy run set-up, extensive integrated features and sample throughput of one to eight samples per run allows the system to meet the demanding nucleic acid isolation needs of research laboratories that have low to medium sample throughput.

The MagNA Pure Compact Instrument utilizes proven magnetic-bead technology to isolate nucleic acids. This technology has been successfully used throughout the world, as shown by numerous publications over the last few years. The choice of nucleic acid purification protocol depends on the sample type and the specific downstream application. The MagNA Pure Compact Instrument incorporates a variety of experimental protocols for different specimen amounts (100 - 1000  $\mu$ l), sample elution volumes (50 - 200  $\mu$ l), and a wide variety of sample materials (blood, tissue, or cultured cells).

The instrument also:

- ▶ Saves time with pre-filled reagents and pre-packaged disposables.
- ▶ Eliminates contamination with an integrated HEPA filter and synchronized stage movement.

High-quality nucleic acids isolated with the MagNA Pure Compact System are suitable for a broad range of applications in nucleic acid research, including gene expression analysis by PCR or RT-PCR (on the LightCycler<sup>®</sup> 2.0 System or on standard block cyclers), microarray analysis, genotyping (on the LightTyper Instrument) and many other downstream applications.

### MagNA Lyser Instrument

The MagNA Lyser Instrument is a compact, benchtop device that offers automated tissue homogenization of up to 16 samples. The device automatically disrupts cells or other biological materials. The instrument simplifies production of a supernatant that contains nucleic acids and proteins; this supernatant is suitable for subsequent purification, extraction, or analysis. For example, supernatants prepared with the MagNA Lyser are suitable for automated nucleic acid isolation with the MagNA Pure LC System or the MagNA Pure Compact System.

During a MagNA Lyser Instrument run, the rotor, which is filled with special sample tubes (MagNA Lyser Green Beads), rapidly oscillates. The oscillation of the instrument agitates the contents of the tubes (ceramic beads, cell material, and lysis reagents) at extremely high speed with a twisting motion.

The cells in the sample tubes are disrupted nearly instantaneously when they collide with the ceramic beads. The rate of collision and energy of impact (both of which determine the effectiveness of the disruption process) depend on the oscillation speed of the instrument and the specific gravity of the beads. By varying both of these parameters, you can ensure optimal disruption of a wide variety of cells. The run time can also be varied to efficiently disrupt different types of tissue.

3

The MagNA Lyser Instrument offers the following labor- and time-saving features:

- ▶ Efficient, consistent, reproducible homogenization of a wide variety of sample materials.
- ▶ Rapid homogenization of up to 16 samples in just a few seconds, with better prevention of nucleic acid degradation.
- ▶ Significantly decreased hands-on time (relative to grinding with mortar and pestle or other manual methods).
- ▶ Easy instrument set-up and clean up, since it uses a removable rotor and pre-filled disposable vials.

## 3.3 Protocols for Isolation of Typical Templates

The protocols in this section highlight the use of various HIGH PURE Kits to prepare template nucleic acids suitable for PCR or RT-PCR.

### 3.3.1 Isolation of High Molecular Weight Nucleic Acids from Cultured Cells with the HIGH PURE PCR Template Preparation Kit

This protocol describes the isolation of high molecular weight nucleic acids from  $10^4$  –  $10^6$  cultured mammalian cells with the HIGH PURE PCR Template Preparation Kit. The purified nucleic acids are suitable for use in standard and long template PCR procedures. If users want an RNA-free preparation of DNA, they may include an optional RNase step at the end of the isolation procedure.



*The HIGH PURE PCR Template Preparation Kit may also be used to isolate nucleic acids from 200 - 300  $\mu$ l whole blood, 200  $\mu$ l buffy coat, 25 – 50 mg mammalian solid tissue, 25 – 35 mg mouse tail,  $10^8$  yeast cells,  $10^9$  bacterial cells or 25 – 50 mg formalin-fixed, paraffin-embedded tissue sections. For details of these procedures, see the package insert for the kit (Cat. No. 11 796 828 001), which is available online at [www.roche-applied-science.com](http://www.roche-applied-science.com).*

#### Additional Equipment and Reagents Required

- ▶ Absolute ethanol
- ▶ Absolute isopropanol
- ▶ Standard, tabletop microfuge capable of  $13,000 \times g$  centrifugal force
- ▶ 1.5 ml microfuge tubes, sterile
- ▶ Phosphate buffered saline (PBS)\*
- ▶ For removal of RNase (optional):
  - ▶ RNase, DNase-free\*
  - ▶ HIGH PURE PCR Product Purification Kit\*
  - ▶ Heating block or water bath, set at 37°C

\* available from Roche Applied Science; see Ordering Information (in the Appendix) for details.

## Overview of Procedure

### Principle of the Kit

Cells are lysed during a short incubation with Proteinase K in the presence of a chaotropic salt (guanidine HCl), which immediately inactivates all nucleases. Cellular nucleic acids bind selectively to glass fiber fleece in a special centrifuge tube. The nucleic acids remains bound while a series of rapid wash-and-spin steps remove contaminating small molecules. Finally, low salt elution removes the nucleic acids from the glass fiber fleece. The process does not require precipitation, organic solvent extractions, or extensive handling of the nucleic acids.

### Time Required

Total time: approx. 20 min

Hands-on time: approx. 12 min

### Yield

15 - 20  $\mu\text{g}$  DNA from  $10^6$  cultured mammalian (K562) cells.

### Purity of Product

Purified nucleic acids are free of DNA polymerase inhibitors and other cellular components.

## Isolation Procedure

### Preliminary Steps

- 1 Prepare all kit reagents for use as detailed in the package insert for the kit.  
 *In the procedures below, all reagents with colored caps are components of the kit.*
- 2 Warm the Elution Buffer (colorless cap, from the kit) to 70°C.

### Sample Lysis and Nucleic Acid Binding

- 1 Centrifuge the culture medium containing  $10^4 - 10^6$  cells, then resuspend the cells in 200  $\mu\text{l}$  PBS.
- 2 Transfer the resuspended cells to a nuclease free 1.5 ml microcentrifuge tube, then:
  - ▶ Add 200  $\mu\text{l}$  Binding Buffer (green cap).
  - ▶ Add 40  $\mu\text{l}$  reconstituted Proteinase K (pink cap).
- 3 Mix the contents of the tube immediately and incubate at 70°C for 10 min.
- 4 Add 100  $\mu\text{l}$  isopropanol and mix well.
- 5
  - ▶ Insert one High Filter Tube into one Collection Tube.
  - ▶ Pipet the entire sample mixture (prepared in Steps 2-4) into the upper buffer reservoir of the Filter Tube.
  - ▶ Insert the entire HIGH PURE Filter Tube assembly into a standard tabletop microfuge.
  - ▶ Centrifuge 1 min at  $8,000 \times g$ .

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## Washing and Elution of Nucleic Acids

- 1** After the initial centrifugation to bind the nucleic acids (above):
  - ▶ Remove the Filter Tube from the Collection Tube; discard the flowthrough liquid and the Collection Tube.
  - ▶ Combine the Filter Tube with a new Collection Tube.
  - ▶ Add 500 µl Inhibitor Removal Buffer (black cap) to the upper reservoir of the Filter Tube.
  - ▶ Insert the entire HIGH PURE assembly into the centrifuge and spin 1 min at  $8,000 \times g$ .
- 2**
  - ▶ Remove the Filter Tube from the Collection Tube; discard the flowthrough liquid and the Collection Tube.
  - ▶ Combine the Filter Tube with a new Collection Tube.
  - ▶ Add 500 µl Wash Buffer (blue cap) to the upper reservoir of the Filter Tube.
  - ▶ Centrifuge 1 min at  $8,000 \times g$  and discard the flowthrough.
- 3**
  - ▶ Remove the Filter Tube from the Collection Tube; discard the flowthrough liquid and the Collection Tube.
  - ▶ Combine the Filter Tube with a new Collection Tube.
  - ▶ Add 500 µl Wash Buffer (blue cap) to the upper reservoir of the Filter Tube.
  - ▶ Centrifuge 1 min at  $8,000 \times g$  and discard the flowthrough.
- 4** After discarding the flowthrough liquid:
  - ▶ Centrifuge the entire HIGH PURE assembly for an additional 10 s at full speed (approx.  $13,000 \times g$ ).
  - ▶ Discard the Collection Tube.
  -  The extra centrifugation time ensures removal of residual Wash Buffer.
- 5** To elute the Nucleic Acids:
  - ▶ Insert the Filter Tube into a clean, sterile 1.5 ml microfuge tube.
  - ▶ Add 200 µl prewarmed ( $70^{\circ}\text{C}$ ) Elution Buffer to the upper reservoir of the Filter Tube.
  - ▶ Centrifuge the tube assembly for 1 min at  $8,000 \times g$ .
- 6** The microfuge tube now contains the eluted nucleic acids, which are suitable for direct use in PCR.
- 7** If you are not going to use all the eluted nucleic acids immediately, store the unused portion at either:
  - ▶  $+2$  to  $+8^{\circ}\text{C}$  for short term storage, or
  - ▶  $-15$  to  $-25^{\circ}\text{C}$  for long term storage.

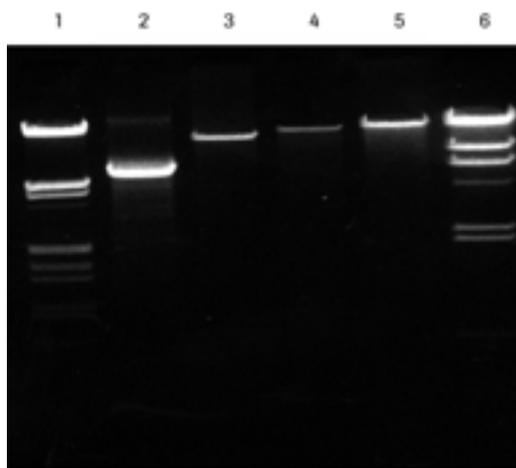
## Removal of RNA from Eluted Nucleic Acids (optional)

The eluted nucleic acids obtained with the HIGH PURE PCR Template Preparation Kit are suitable for direct use in many PCRs. However, if you wish to further purify the preparation by removing RNA from it before PCR, you may treat the eluted nucleic acids as follows:

- 1**
  - ▶ To the eluted nucleic acids from  $10^6$  cultured mammalian cells, add 0.5 µl of DNase-free RNase.
  - ▶ Incubate the cells for 15 min at either:
    - ▶  $+15$  to  $+25^{\circ}\text{C}$ , or
    - ▶  $37^{\circ}\text{C}$
- 2** Remove the RNase from the purified DNA with the HIGH PURE PCR Product Purification Kit
  -  For details on the HIGH PURE PCR Product Purification Kit, see Chapter 6.

### Typical Results with the Kit

**Result:** All nucleic acid preparations yielded a distinct, specific band of the expected fragment length. Thus, the HIGH PURE PCR Template Preparation Kit produces high quality template suitable for amplification of long targets.



**Figure 3.3.1. Amplification of a single copy human tPA gene from human nucleic acids prepared with the HIGH PURE PCR Template Preparation Kit.** Nucleic acids were purified from whole human blood or cultured human K562 cells. Various sequences from the human tPA gene were amplified from aliquots (250 ng) of the nucleic acid preparations with either the Expand Long Template or Expand 20 kb<sup>PLUS</sup> PCR System. Different tPA primers were used for each reaction; the primer annealing temperature for all reactions was 65°C. The PCR cycling conditions and the sequence of the tPA primers were as described in the Expand Long Template and 20 kb<sup>PLUS</sup> PCR System package inserts. Products were separated on an agarose gel.

**Lane 1 and 6,** molecular weight markers. The amplicons obtained were:

**Lane 2:** 6.3 kb tPA fragment; obtained from blood nucleic acids, amplified in Expand Long Template PCR buffer 1.

**Lane 3:** 15 kb tPA fragment; obtained from blood nucleic acids, amplified in Expand Long Template PCR buffer 3.

**Lane 4:** 23 kb tPA fragment; obtained from blood nucleic acids, amplified in Expand Long Template PCR buffer 3.

**Lane 5:** 28 kb tPA fragment; obtained from K562 nucleic acids; amplified with the Expand 20 kb<sup>PLUS</sup> PCR System.

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### 3.3.2 Isolation of Total RNA from Whole Blood with the HIGH PURE RNA Isolation Kit

This protocol describes the isolation of intact total RNA from 500 µl human whole blood with the HIGH PURE RNA Isolation Kit. The purified RNA is suitable for use in RT-PCR procedures.



*The HIGH PURE RNA Isolation Kit may also be used to isolate total RNA from smaller amounts (as little as 200 µl) whole blood, 10<sup>6</sup> cultured mammalian cells, 10<sup>8</sup> yeast cells, or 10<sup>9</sup> bacterial cells. For details of these procedures, see the package insert for the kit (Cat. No. 11 828 665 001), which is available online at [www.roche-applied-science.com](http://www.roche-applied-science.com).*

#### Additional Equipment and Reagents Required

- ▶ Absolute ethanol
- ▶ Standard, tabletop microfuge capable of 13,000 × g centrifugal force
- ▶ 1.5 ml microfuge tubes, sterile
- ▶ Phosphate buffered saline (PBS)\*
- ▶ Red Blood Cell Lysis Buffer\*

\* available from Roche Applied Science; see Ordering Information (in the Appendix) for details.

## Overview of Procedure

### Principle of the Kit

A single reagent lyses the sample and inactivates RNase. In the presence of a chaotropic salt (guanidine HCl), the released total RNA binds selectively to glass fiber fleece in a special centrifuge tube. The RNA remains bound while a DNase treatment and a series of rapid wash-and-spin steps remove DNA and contaminating small molecules. Finally, low salt elution removes the RNA from the glass fiber fleece. The process does not require precipitation, organic solvent extractions, or extensive handling of the RNA.

### Time Required

Total time: approx. 25 min (+ pretreatment to lyse red blood cells)

Hands-on time: less than 10 min

### Purity of Product

Purified RNA is free of DNA, protein, salts and other cellular components.

### Isolation Procedure



*For this procedure, use only fresh, whole blood that has been preserved with EDTA.*

### Preliminary Step: Lysis of Red Blood Cells with Red Blood Cell Lysis Buffer

- 1 Add 1 ml Red Blood Cell Lysis Buffer to a sterile 1.5 ml microfuge tube.
- 2 Add 500 µl human whole blood (preserved with EDTA) to the tube and mix by inversion.
  - Do not vortex.*
- 3 Either:
  - ▶ Place the tube on a rocking platform or gyratory shaker for 10 min at +15 to +25° C, or
  - ▶ Manually invert the sample periodically for 10 min.
- 4 Centrifuge for 5 min at 500 × *g* in a standard tabletop microfuge.
- 5
  - ▶ With a pipette, carefully remove and properly dispose of the clear, red supernatant.
  - ▶ Add 1 ml Red Blood Cell Lysis Buffer to the white pellet and mix by “flicking” the tube until the pellet is resuspended.
  - Do not vortex.*
- 6
  - ▶ Centrifuge for 3 min at 500 × *g*.
  - ▶ Carefully remove and properly dispose of the supernatant, particularly the red ring of blood cell debris that forms around the outer surface of the white pellet.
- 7 Resuspend the white pellet in 200 µl PBS and use the resuspended sample in the lysis/binding procedure below.

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### Preparation of Total RNA with the HIGH PURE RNA Isolation Kit

 In the procedures below, all reagents with colored caps are components of the kit. Before starting the procedure, prepare all kit reagents for use as detailed in the package insert for the kit.

- 1** ▶ To the 200  $\mu$ l sample (in PBS) prepared in the procedure above, add 400  $\mu$ l Lysis/Binding Buffer (green cap).  
▶ Vortex the tube for 15 seconds.
- 2** ▶ Insert one High Filter Tube in one Collection Tube.  
▶ Pipet the entire mixture from Step 1 into the upper buffer reservoir of the Filter Tube.  
▶ Insert the entire HIGH PURE Filter Tube assembly into a standard tabletop microfuge.  
▶ Centrifuge 15 seconds at  $8,000 \times g$ .
- 3** ▶ Remove the Filter Tube from the Collection Tube; discard the flowthrough liquid.  
▶ Again combine the Filter Tube and the used Collection Tube.
- 4** ▶ Add 90  $\mu$ l DNase Incubation Buffer (white cap) to a clean, sterile 1.5 ml microfuge tube, then add 10  $\mu$ l reconstituted DNase I (from the kit) to the buffer.  
▶ Pipet the diluted DNase I solution directly onto the glass fiber fleece in the upper reservoir of the Filter Tube (from Step 3 above).  
▶ Incubate the entire Filter Tube assembly for 15 min at +15 to +25°C.
- 5** ▶ Add 500  $\mu$ l Wash Buffer I (black cap) to the upper reservoir of the Filter Tube assembly.  
▶ Centrifuge 15 seconds at  $8,000 \times g$  and discard the flowthrough.  
▶ Again combine the Filter Tube and the used Collection Tube.
- 6** ▶ Add 500  $\mu$ l Wash Buffer II (blue cap) to the upper reservoir of the Filter Tube assembly.  
▶ Centrifuge 15 seconds at  $8,000 \times g$  and discard the flowthrough.  
▶ Again combine the Filter Tube and the used Collection Tube.
- 7** ▶ Add 200  $\mu$ l Wash Buffer II (blue cap) to the upper reservoir of the Filter Tube assembly.  
▶ Centrifuge 2 min at maximum speed (approx.  $13,000 \times g$ ).  
 *This high speed centrifugation step ensures removal of any residual Wash Buffer.*
- 8** ▶ Discard the used Collection Tube and any flowthrough liquid it contains.  
▶ Insert the Filter Tube into a clean, sterile 1.5 ml microfuge tube.
- 9** To elute the RNA:  
▶ Add 50 – 100  $\mu$ l Elution Buffer to the upper reservoir of the Filter Tube.  
▶ Centrifuge the tube assembly for 1 min at  $8,000 \times g$ .
- 10** The microfuge tube now contains the eluted RNA, which is suitable for direct use in RT-PCR.  
 *Due to the modest amount of RNA in leukocytes, we recommend that the isolated RNA be used exclusively in one-step or two-step RT-PCR, not in other procedures.*
- 11** If you are not going to use all the eluted RNA immediately, store the unused portion at  $-80^{\circ}\text{C}$  for later analysis.

# 3

### 3.3.3 Isolation of cDNA with the HIGH PURE PCR Product Purification Kit

The HIGH PURE PCR Product Purification Kit protocol described below can also be used for the isolation of first strand cDNA, e.g. after reverse transcription of total RNA (Footitt *et al.*, 2003).



The principle use for the HIGH PURE PCR Product Purification Kit is isolation of amplified DNA products that are at least 100 bp (and less than 50 kb) long. (See Chapter 6 for more information on this application.) The kit may also be used to isolate enzymatically labeled, modified or digested DNA [e.g., products from restriction digests (Lobner *et al.*, 2002), alkaline phosphatase treatments, kinase reactions, or other enzymatic reactions (Chang *et al.*, 2001; Salesse and Verfaillie, 2003)], DNA from a 100 mg agarose gel slice, and RNA from *in vitro* transcription reactions. For details on these procedures, see the package insert for the kit (Cat. No. 11 732 668 001), which is available online at [www.roche-applied-science.com](http://www.roche-applied-science.com).

#### Additional Equipment and Reagents Required

- ▶ Absolute ethanol
- ▶ Standard, tabletop microfuge capable of 13,000 × g centrifugal force
- ▶ 1.5 ml microfuge tubes, sterile
- ▶ Water, PCR Grade\* or double dist. Water

\* available from Roche Applied Science; see Ordering Information (in the Appendix) for details.

#### Overview of Procedure

##### Principle of the Kit

In the presence of chaotropic salt, DNA binds selectively to glass fiber fleece in a special centrifuge tube. The cDNA remains bound while a series of rapid wash-and-spin steps remove contaminating small molecules (including small nucleic acids). Finally, low salt elution removes the DNA from the glass fiber fleece. The process does not require DNA precipitation, organic solvent extractions, or extensive handling.

##### Time Required

Total time: approx. 10 min

Hands-on time: less than 10 min

## Purity of Product

Purified DNA is free of short DNA (<100 bp), small molecules (e.g., primers, salts, unincorporated nucleotides) and proteins (e.g., reverse transcriptase).

## Isolation Procedure



*In the procedures below, all reagents with colored caps are components of the kit. Before starting the procedure, prepare the Wash Buffer solution for use (by adding absolute ethanol) as detailed in the package insert for the kit.*

- 1**
  - ▶ Adjust the volume of the DNA sample to 100 µl with Water, PCR grade\* or double dist. water.
  - ▶ Add 500 µl Binding Buffer (green cap) to the sample tube.
  - ▶ Mix the contents of the tube well.
- 2**
  - ▶ Insert one High Filter Tube into one Collection Tube.
  - ▶ Pipet the entire mixture from Step 1 into the upper buffer reservoir of the Filter Tube.
  - ▶ Insert the entire HIGH PURE Filter Tube assembly into a standard tabletop microfuge at +15 to +25°C.
  - ▶ Centrifuge 30 – 60 seconds at maximum speed (approx. 13,000 × g).
- 3**
  - ▶ Remove the Filter Tube from the Collection Tube; discard the flowthrough liquid.
  - ▶ Again combine the Filter Tube and the used Collection Tube.
- 4**
  - ▶ Add 500 µl Wash Buffer (blue cap) to the upper reservoir of the Filter Tube assembly.
  - ▶ Centrifuge 1 min at maximum speed (as above).
  - ▶ Remove the Filter Tube from the Collection Tube; discard the flowthrough liquid.
  - ▶ Again combine the Filter Tube and the used Collection Tube.
- 5**
  - ▶ Add 200 µl Wash Buffer (blue cap) to the upper reservoir of the Filter Tube assembly.
  - ▶ Centrifuge 1 min at maximum speed (as above).
  -  *This high speed centrifugation step ensures optimal purity and complete removal of any residual Wash Buffer.*
- 6**
  - ▶ Discard the used Collection Tube and any flowthrough liquid it contains.
  - ▶ Insert the Filter Tube into a clean, sterile 1.5 ml microfuge tube.
- 7**
  - ▶ To elute the DNA:
    - ▶ Add 50 – 100 µl Elution Buffer (colorless cap) to the upper reservoir of the Filter Tube.
    - ▶ Centrifuge the tube assembly 1 min at maximum speed.
    -  *Do not use water for elution, since an alkaline pH is required for optimal yield.*
- 8**
  - ▶ The microfuge tube now contains the eluted DNA, which is suitable for direct use.
  -  *If you plan to estimate the DNA yield by determining its  $A_{260}$ , residual glass fibers in the eluate may interfere. To remove these glass fibers, centrifuge the tube containing the eluate at maximum speed for longer than 1 min. Then use only an aliquot of the eluate for the absorbance measurement.*

# 3

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# 3

*Chapter 4*

**General PCR Methods**



**4**

4	General PCR Methods	Page
	PCR Protocol Selection Guide.....	65
4.1	Basic PCR.....	66
4.1.1	Hot Start PCR - The new Standard.....	66
4.1.1.1	Reagents and Equipment Required.....	66
4.1.1.2	General Considerations for Hot Start PCR.....	67
4.1.1.3	Protocols for Hot Start PCR (for Targets up to 3 kb).....	67
4.1.1.4	Typical Results.....	73
4.1.2	Conventional PCR.....	74
4.1.2.1	Reagents and Equipment Required.....	74
4.1.2.2	General Considerations for Conventional Standard PCR.....	75
4.2	High Fidelity PCR.....	79
4.2.1	Reagents and Equipment Required.....	79
4.2.2	General Considerations for High Fidelity PCR.....	80
4.2.3	Protocols for High Fidelity PCR.....	81
4.2.4	Typical Results.....	90
4.3	Long Template PCR.....	92
4.3.1	Reagents and Equipment Required.....	92
4.3.2	General Considerations for Long Template PCR.....	93
4.3.3	Protocols for Long Template PCR.....	94
4.4	Amplification of Difficult Templates.....	101
4.4.1	Reagents and Equipment Required.....	101
4.4.2	General Considerations for Amplification of Difficult Templates.....	102
4.4.3	Protocols for Amplification of Difficult Templates (for Targets up to 5 kb).....	103
4.4.4	Typical Results.....	107
4.5	Guidelines for Optimizing PCR.....	108
4.5.1	Reagents and Equipment Required.....	108
4.5.2	General Considerations for Preventing Carryover.....	109
4.5.3	Protocols for Preventing Carryover.....	110
4.6	Preventing Carryover.....	117
4.6.1	Choose the Appropriate Enzyme.....	117
4.6.2	Use Highly Purified Templates and Primers.....	117
4.6.3	Design Primers Carefully.....	117
4.6.4	Use the Highest Quality Nucleotides.....	118
4.6.5	Minimize Pipetting Steps with Convenient Master Reagent Mixes.....	119
4.6.6	Optimize the Reaction Components.....	120
4.6.7	Optimize Reaction Temperatures and Times.....	123

# 4

## 4. General PCR Methods

### PCR Protocol Selection Guide

The path to optimal PCR results starts with choosing the correct PCR enzyme system from Roche Applied Science and the optimal PCR protocol (one designed specifically for the enzyme and your template). For optimal PCR results, follow the guidelines in the table below:



To Amplify Genomic DNA, Plasmid DNA or cDNA with These Characteristics...	And Make Products...	Choose this Roche Applied Science Enzyme System	And Use this PCR Protocol (page in this chapter)
Average GC content, target available in abundant quantities	With high yield		
	up to 3 kb long	FastStart Taq DNA Polymerase	Basic PCR, Hot Start, protocol A (page 68)
	up to 3 kb long	Taq DNA Polymerase	Basic PCR, Conventional, protocol B (page 70)
	up to 5 kb long	Expand High Fidelity PCR System	High Fidelity PCR, protocol F (page 86)
	5–25 kb long	Expand Long Range dNTPack	Long Template PCR, protocol G (page 94)
	20–35 kb long	Expand 20 kb <sup>PLUS</sup> PCR System	Long Template PCR, protocol H (page 96)
	With great accuracy <sup>a</sup>		
	up to 3 kb long	Pwo SuperYield DNA Polymerase	High Fidelity PCR, protocol D (page 81)
	up to 5 kb long	FastStart High Fidelity PCR System	High Fidelity PCR, protocol E (page 83)
	up to 5 kb long	Expand High Fidelity PCR System	High Fidelity PCR, protocol F (page 86)
	Free of carryover contaminants <sup>b</sup>		
	up to 3 kb long	PCR Core Kit <sup>PLUS</sup>	Preventing Carryover, protocol L (page 110)
up to 5 kb long	Expand High Fidelity <sup>PLUS</sup> PCR System	Preventing Carryover, protocol M (page 113)	
Target available in limited quantities	up to 3 kb long	Pwo SuperYield DNA Polymerase	High Fidelity PCR, protocol D (page 81)
	up to 5 kb long	FastStart High Fidelity PCR System	High Fidelity PCR, protocol E (page 83)
	up to 5 kb long	Expand High Fidelity PCR System	High Fidelity PCR, protocol F (page 86)
High GC content	up to 3 kb long	FastStart Taq DNA Polymerase	Hot Start PCR, protocol C (page 76)
	up to 5 kb long	GC-RICH PCR System	Amplification of Difficult Templates, protocol I, K, (page 103, 105)
	5–25 kb long	Expand Long Range dNTPack	Long Template PCR, protocol G (page 94)
Repetitive sequences, complex sequence mixtures (e.g., for multiplexing) or other sequences that are difficult to amplify	up to 3 kb long	FastStart Taq DNA Polymerase	Hot Start PCR, protocol C (page 76)
	up to 5 kb long	GC-RICH PCR System	Amplification of Difficult Templates, protocol I, K, (page 103, 105)
	up to 5 kb long	FastStart High Fidelity PCR System	High Fidelity PCR, protocol E (page 83) Optimizing Multiplex PCR (Chapter 8)
	5–25 kb long	Expand Long Range dNTPack	Long Template PCR, protocol G (page 94)

<sup>a)</sup> e.g., for sequencing or mutation studies

<sup>b)</sup> e.g., to eliminate false negatives in qualitative assays

## 4.1 Basic PCR

FastStart Taq DNA Polymerase is the best PCR enzyme for most up-to-date standard PCRs. Combining high quality with very high sensitivity, FastStart Taq DNA Polymerase produces the best results. The enzyme is inactive at temperatures up to 75°C, but readily activated at DNA denaturing temperatures. This so-called “hot start” enzyme thus minimize the formation of troublesome primer-dimers during reaction set-up and PCR.

Besides FastStart Taq DNA Polymerase, Roche Applied Science still offers the conventional non-hot start Taq DNA Polymerase. For many routine, classical PCRs, Taq DNA Polymerase is still a convenient PCR enzyme. High-quality, recombinant Taq DNA Polymerase (such as the preparation available from Roche Applied Science) produces the best results.

### 4.1.1 Hot Start PCR - The new Standard

#### Purpose of Procedure:

Amplification of normal and GC-rich templates up to 3 kb in length

#### Enzyme/System Needed:

FastStart Taq DNA Polymerase (for templates up to 3 kb)



*For hot start amplification of longer templates (up to 5 kb), use the FastStart High Fidelity PCR System (described in section 4.2).*



*For more information on choosing the correct amplification procedure, see the PCR Protocol Selection Guide at the beginning of Chapter 4.*

#### 4.1.1.1 Reagents and Equipment Required

#### PCR Enzyme Required for this Protocol:

FastStart Taq DNA Polymerase



*You can also perform hot start PCR with the FastStart PCR Master, a ready-to-use, 2× reaction mixture (containing FastStart Taq DNA Polymerase, buffer, nucleotides and MgCl<sub>2</sub>). This convenient product lets you prepare complete hot start PCR mixes with fewer pipetting steps. For a detailed hot start protocol that uses the FastStart PCR Master, see the package insert of this product, available online at [www.roche-applied-science.com](http://www.roche-applied-science.com).*

*For details on the characteristics of FastStart Taq DNA Polymerase, see Chapter 2. For pack sizes, see Ordering Information in the Appendix.*

### Additional Equipment and Reagents Required

- ▶ Thermal block cycler (e.g., Applied Biosystems GeneAmp PCR System 2400) or other PCR instrument
- ▶ 0.2 ml thin-walled PCR tubes\* or PCR microplates, suitable for your PCR instrument
- ▶ Sterile 1.5 ml reaction tubes for preparing master mixes and dilutions
- ▶ PCR Grade Nucleotide Mix\* (10 mM of each nucleotide)
  -  *The PCR Grade Nucleotide Mix is available either separately or as part of a convenient enzyme-dNTPack (e.g., FastStart Taq DNA Polymerase, dNTPack\*)*
- ▶ PCR Grade Water \*

\* available from Roche Applied Science; see Ordering Information (in the Appendix) for details.

#### 4.1.1.2 General Considerations for Hot Start PCR

##### Sample Material

Any suitable target DNA up to 3 kb in length, including GC-rich DNA

 *As with any PCR, the quality of the template influences the outcome of the PCR. For more information on preparing high quality DNA templates, see Chapter 3.*

##### Template Dilution Buffer

The optimal dilution buffer for template DNA is either sterile, double-distilled water or Tris (5–10 mM, pH 7–8).

 *Do not dissolve the template in TE buffer because EDTA chelates  $Mg^{2+}$ .*

#### 4.1.1.3 Protocols for Hot Start PCR (for Targets up to 3 kb)

 *For “Hot Start” amplification of longer templates (up to 5 kb), use protocol E in section 4.2, “High Fidelity PCR.”*

## Protocol A: Hot Start Amplification of Normal Templates (up to 3 kb)

### Setting Up the Reaction

#### Setting Up the Reaction

- 1 ▶ Thaw all frozen reagents before use.  
▶ Mix all reagents thoroughly and briefly centrifuge them before starting the procedure.
- 2 Prepare the master mix in a sterile 1.5 ml reaction tube (on ice).  
 Add the components in the order listed below. Numbered vials are supplied with the enzyme.

#### Master Mix (for one reaction) <sup>a</sup>

Components	Volume	Final conc.
PCR Grade water	variable	to make final volume (including template) = 50 µl
PCR reaction buffer with MgCl <sub>2</sub> , 10× (vial 2)		2 mM MgCl <sub>2</sub>
PCR Grade Nucleotide Mix, 10 mM	1 µl	200 µM (each nucleotide)
Downstream primer	variable	200 nM
Upstream primer	same as downstream primer	200 nM
FastStart Taq DNA Polymerase (vial 1)	0.4 µl	2 U
<p>▶ Mix all the above ingredients thoroughly and transfer the whole mix to a PCR tube or well of a microplate. If you prepared Master Mix for more than one reaction<sup>a</sup>, pipet equal volumes of it into separate PCR tubes.</p> <p>▶ Add template DNA to the individual tubes (or wells) containing Master Mix, using the following guidelines:</p>		
DNA or cDNA template	variable	10 pg–500 ng complex DNA 10 pg–100 ng cDNA 10 pg–100 ng plasmid DNA
<b>Final volume (including template)</b>	<b>50 µl</b>	<b>1× Reaction Mix</b>

<sup>a)</sup> To prepare a Master Mix for more than one reaction, multiply the amounts of the first 6 ingredients (as listed under “Volume”) by Z, where Z = the number of reactions to be run + one additional reaction.

- 3 ▶ Gently mix the solutions in each PCR tube (or well) to produce a homogeneous reaction mixture.  
▶ According to the instructions supplied with your instrument, prepare the tubes or microplate for PCR (e.g., overlay reaction mixture in tube with mineral oil or seal the microplate with adhesive foil).
- 4 Place PCR tubes (or microplate) in the PCR instrument and begin PCR immediately.  
 The completed reaction mixtures should not be stored for extended periods of time.

4

## PCR

After placing the samples in the PCR instrument, cycle according to the profile below.

 *The thermal profile below was developed for the Applied Biosystems GeneAmp PCR System 2400. Other thermal block cyclers may require a different profile.*

Cycles		Temperature	Time
<b>Denaturation/Activation</b>			
1		95°C	4 min <sup>a</sup>
<b>Amplification Program</b>			
30 – 40 <sup>b,c</sup>	Denaturation	95°C	30 s
	Annealing	45 to 65°C <sup>d</sup>	30 s
	Elongation	72°C	45 s–3 min <sup>e</sup>
<b>Final Elongation</b>			
1		72°C	7 min

- <sup>a)</sup> This step serves two purposes. It activates the FastStart Taq DNA Polymerase as well as denaturing the template. For specific applications or to increase yields, you may vary the activation time from 2 min (for sensitive templates) to 10 min (for complex mixtures, *e.g.*, for multiplexing up to 14 bands with 28 primers).
- <sup>b)</sup> Thirty (total) cycles is usually enough to produce an adequate amount of product if the starting template contains  $>10^4$  copies of the target. For low concentrations of target or complex (*e.g.*, multiplexing) target mixtures, increase the total number of cycles to 40.
- <sup>c)</sup> Gradually increasing elongation time ensures a higher yield of amplification products. To increase the yield of amplification products, modify the amplification program so the elongation time increases gradually (*e.g.*, by 5 seconds/cycle) after 15 cycles of amplification, (For details, see the FastStart Taq DNA Polymerase package insert.)
- <sup>d)</sup> Optimal annealing temperature depends on the melting temperature of the primers and the system used.
- <sup>e)</sup> Elongation time depends on fragment length. Use 1 min/kb for fragments up to 3 kb.

4

**Protocol B: Hot Start Amplification of GC-rich Templates (up to 3 kb)****Setting Up the Reaction**

- 1 ▶ Thaw all frozen reagents before use.  
▶ Mix all reagents thoroughly and briefly centrifuge them before starting the procedure.
- 2 Prepare the master mix in a sterile 1.5 ml reaction tube (on ice).  
 *Add the components in the order listed below. Numbered vials are supplied with the enzyme.*

**Master Mix** (for one reaction)<sup>a</sup>

Components	Volume	Final conc.
PCR Grade water	variable	
PCR reaction buffer with MgCl <sub>2</sub> , 10× (vial 2)		2 mM MgCl <sub>2</sub>
GC-RICH solution, 5× (vial 5)	5 µl	1×
PCR Grade Nucleotide Mix, 10 mM	1 µl	200 µM (each nucleotide)
Downstream primer	variable	200 nM
Upstream primer	same as downstream primer	200 nM
FastStart Taq DNA Polymerase (vial 1)	0.4 µl	2 U
▶ Mix the first 7 ingredients thoroughly and transfer the whole mix to a PCR tube or well of a microplate. If you prepared Master Mix for more than one reaction <sup>a</sup> , pipet equal volumes of it into separate PCR tubes. ▶ Add template DNA to the individual tubes (or wells) containing Master Mix, using the following guidelines:		
DNA or cDNA template	variable	10 pg–500 ng complex DNA 10 pg–100 ng cDNA 10 pg–100 ng plasmid DNA
<b>Final volume (including template)</b>	<b>50 µl</b>	1× Reaction Mix

<sup>a)</sup> To prepare a Master Mix for more than one reaction, multiply the amounts of the first 7 ingredients (as listed under "Volume") by Z, where Z = the number of reactions to be run + one additional reaction.

- 3 ▶ Gently mix the solutions in each PCR tube (or well) to produce a homogeneous reaction mixture.  
▶ According to the instructions supplied with your instrument, prepare the tubes or microplate for PCR (*e.g.*, overlay reaction mixture in tube with mineral oil or seal the microplate with adhesive foil).
- 4 Place PCR tubes (or microplate) in the PCR instrument and begin PCR immediately.  
 *The completed reaction mixtures should not be stored for extended periods of time.*

**PCR**

Use the same PCR program as in Protocol A above.

4

## Troubleshooting the Amplification

### Optimizing Reaction Conditions

For initial experiments, use the conditions listed in the protocols above, which generally give good results. However, if initial results are unsatisfactory, use the following guidelines to optimize the critical components of the reaction:

Parameter	Optimal Concentration or Condition
Template DNA	10 pg - 500 ng complex DNA (e.g., human genomic DNA) 10 pg - 100 ng cDNA or plasmid
FastStart Taq DNA Polymerase	between 2.0 and 5.0 U per 50 µl reaction
Primers	between 200 and 500 nM (final concentration of each primer)
dNTPs	between 100 and 500 µM
MgCl <sub>2</sub>	between 1 and 4 mM <sup>a</sup>

<sup>a)</sup> For optimization, use the PCR reaction buffer without MgCl<sub>2</sub> (vial 3, supplied with enzyme) and titrate the Mg<sup>2+</sup> concentration in 0.5 mM steps. Use the 25 mM MgCl<sub>2</sub> solution (vial 4, supplied with enzyme) to produce the following concentrations:

#### If you add the following volume of 25 mM MgCl<sub>2</sub> stock to a 50 µl (final volume) PCR mixture:

2 µl      3 µl      4 µl      5 µl      6 µl      7 µl      8 µl

#### You will end up with a final MgCl<sub>2</sub> reaction concentration of:

1 mM      1.5 mM      2 mM      2.5 mM      3 mM      3.5 mM      4 mM

 *Optimal reaction conditions (reagent concentrations, incubation times and temperatures, etc.) vary from system to system. For general information on optimizing a PCR reaction, see section 4.7, “Guidelines for Optimizing PCR.”*

### More Troubleshooting Options

- ▶ If Protocol A does not give the expected yield or specificity with the template, try using Protocol B (with GC-RICH Solution).
- ▶ If initial experiments with Protocol B produce nonspecific amplification products, repeat the protocol but increase the amount of the GC-RICH Solution in 5 µl steps, up to a maximum of 25 µl.
- ▶ If either protocol gives little or no PCR product, try the following (in order listed):
  - ▶ Check quality of template, primers and reagents.
  - ▶ Activate FastStart Taq DNA Polymerase for longer times (up to 10 min).
  - ▶ Titrate the concentration of Mg<sup>2+</sup>. See “Optimizing Reaction Conditions” above.
  - ▶ Titrate the concentrations of polymerase and primers.
  - ▶ Optimize cycling conditions.



*For additional troubleshooting options, see “Troubleshooting” in the Appendix of this manual.*



## Processing of PCR Products

### Analysis

Agarose gel electrophoresis is the easiest and commonest way of separating and analyzing DNA. The gel might be used to view or quantify the DNA, or to isolate a particular band. The DNA is visualized in the gel by the addition of ethidium bromide. This binds strongly to DNA by intercalating between the bases and is fluorescent, *i.e.*, it absorbs invisible UV light and transmits the energy as visible orange light.

Analyze the products on a 1 - 2% agarose gel (depending on the length of PCR products.) Use a TAE or TBE buffer system; the percentage of the gel and the buffer system depend on the length of PCR fragments.

Most agarose gels are made between 0.7% and 2%. A 0.7% gel will show good separation (resolution) of large DNA fragments (5–10 kb) and a 2% gel will show good resolution for small fragments (0.2–1 kb). While you can also prepare 3% gels for separating very tiny fragments, a vertical polyacrylamide gel is more appropriate in this case.

Please note that low percentage gels are very weak and may break when you try to lift them. High-percentage gels may be brittle and do not set evenly. 1% gels should usually be prepared.

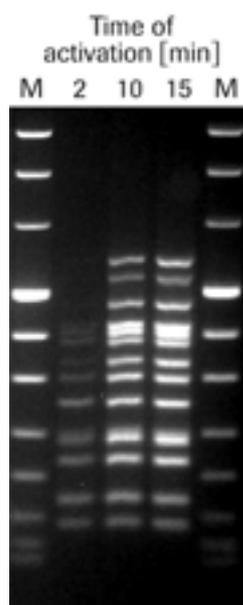
### Cloning

We recommend TA cloning. PCR products generated with FastStart Taq DNA Polymerase usually have a single A overhang (3' end).



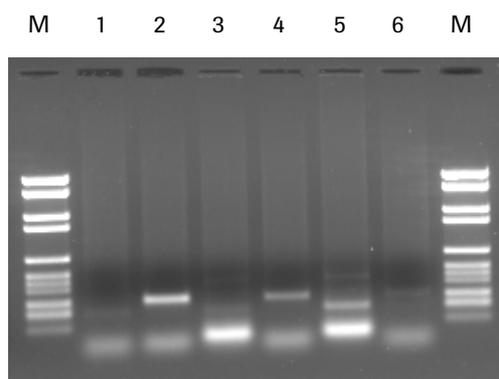
#### 4.1.1.4 Typical Results

**Result:** After adequate activation, FastStart Taq DNA Polymerase produces specific amplicons from all 14 targets with approximately equivalent yield. Thus, FastStart Taq DNA Polymerase can be used for difficult amplifications, even for challenging multiplex PCRs.



**Figure 4.1.1. Simultaneous amplification of 14 targets with FastStart Taq DNA Polymerase.** Selected parts of the gene for the human cystic fibrosis transmembrane conductance regulator (CFTR) were amplified with the FastStart Taq DNA Polymerase and 14 pairs of PCR primers. The amplicon lengths varied from 198 to 598 bp. The results show the need for adequate activation of the FastStart enzyme, since optimal amplification was obtained only after the enzyme was activated for at least 10 min. M = DNA Molecular Weight Marker VIII.

**Result:** The upper band seen in lanes 2 and 4 is the only amplicon of the correct size (based on the size of the target). Therefore, the combination of FastStart Taq DNA Polymerase and GC-RICH Solution (Lane 2) is the only enzyme/buffer combination that can accurately amplify the GC-rich template and still produce a good product yield.



**Figure 4.1.2. Amplification of a GC-rich template with different hot start enzyme preparations and buffers.**

A 284 bp ApoE fragment (GC content, 74%) was amplified from 200 ng of human genomic DNA with different hot start enzyme preparations. Products from each reaction were analyzed on an agarose gel. A molecular weight marker was included at either end of the gel to provide size reference markers. All enzyme preparations were used according to instructions from the enzyme supplier.

The enzyme preparations used were:

**Lane 1:** FastStart Taq DNA Polymerase, with normal reaction buffer (Protocol A above)

**Lane 2:** FastStart Taq DNA Polymerase, with GC-RICH Solution (Protocol B above)

**Lane 3:** Hot start polymerase from another supplier, with standard buffer from the supplier

**Lane 4:** Hot start polymerase from the same supplier as in lane 3, but with a special buffer from the supplier

**Lane 5:** Hot start polymerase from a third supplier, with standard buffer from the supplier

**Lane 6:** Taq DNA Polymerase (not a hot start enzyme)

## 4.1.2 Conventional PCR

### Purpose of Procedure:

Amplification of normal DNA templates up to 3 kb in length

### Enzyme/System Needed:

Taq DNA Polymerase



For more information on choosing the correct amplification procedure, see the PCR Protocol Selection Guide at the beginning of Chapter 4.

### 4.1.2.1 Reagents and Equipment Required

#### PCR Enzyme Required for This Protocol: Taq DNA Polymerase, 1 U/μl



Roche Applied Science has several other Taq DNA Polymerase preparations that may be used to perform standard PCR. For example, we also package Taq DNA Polymerase at different concentrations (e.g., 5 U/μl) and in different grades (e.g., GMP Grade).

You can also perform conventional standard PCR with either of two convenient reagent combinations, the PCR Master or the PCR Core Kit. For detailed standard PCR protocols that use these products, see the following pack inserts:

Product	Purpose	For a complete protocol, see the pack insert for
PCR Master	Ready-to-use, 2× reaction mix (containing Taq DNA Polymerase, buffer, nucleotides, MgCl <sub>2</sub> ) for preparing complete PCR mixes with fewer pipetting steps	Cat. No. 11 636 103 001 <sup>a</sup>
PCR Core Kit	All individual reagents required for PCR except template and primer; no other reagents to buy	Cat. No. 11 578 553 001 <sup>a</sup>

<sup>a)</sup> Available online at [www.roche-applied-science.com](http://www.roche-applied-science.com).



For details on the characteristics of Taq DNA Polymerase, see Chapter 2. For pack sizes of the various products for standard PCR, see Ordering Information in the Appendix.

### Additional Equipment and Reagents Required

- ▶ Thermal block cycler (e.g., Applied Biosystems GeneAmp PCR System 2400) or other PCR instrument
- ▶ 0.2 ml thin-walled PCR tubes\* or PCR microplate, suitable for your PCR instrument
- ▶ Sterile reaction tubes for preparing master mixes and dilutions
- ▶ PCR Grade Nucleotide Mix\* (10 mM of each nucleotide)
  -  *The PCR Grade Nucleotide Mix is available either separately or as part of a convenient enzyme-dNTPack (e.g., Taq DNA Polymerase, dNTPack\*)*
- ▶ PCR Grade Water \*

\* available from Roche Applied Science; see Ordering Information (in the Appendix) for details.

 *The package of Taq DNA Polymerase, 1 U/μl, also includes two PCR reaction buffers (± Mg<sup>2+</sup>) and a 25 mM MgCl<sub>2</sub> stock solution.*

#### 4.1.2.2 General Considerations for Conventional Standard PCR

##### Sample Material

Taq DNA Polymerase will amplify any suitable DNA up to 3 kb in length. DNA samples should be available in μg quantities, have an average GC content, no unusual sequences, and be as pure as possible.

 *As with any PCR, the quality of the template influences the outcome of the PCR. For more information on preparing high quality DNA templates, see Chapter 3 of this manual.*

##### Template Dilution Buffer

The optimal dilution buffer for template DNA is either sterile, double-distilled water or Tris (5–10 mM, pH 7–8).

 *Do not dissolve the template in TE buffer because EDTA chelates Mg<sup>2+</sup>.*

## Protocol C: Conventional Standard PCR

### Amplification Procedure

#### Setting Up the Reaction

- 1 ▶ Thaw all frozen reagents before use.  
▶ Mix all reagents and briefly centrifuge them before starting the procedure.
- 2 Prepare two separate master mixes in sterile 1.5 ml microfuge tubes (on ice). (Master Mix 1 contains dNTPs, primers, and template DNA; Master Mix 2 contains PCR reaction buffer and enzyme.)

 For each mix, add the components in the order listed below.

#### Master Mix 1 (for one reaction)

Components (Mix 1)	Volume <sup>a</sup>	Final conc.
PCR Grade water	variable	
PCR Grade Nucleotide Mix, 10 mM	1 µl	200 µM (each nucleotide)
Downstream primer	variable	200 nM
Upstream primer	same as downstream primer	200 nM
Template DNA	variable	up to 200 ng genomic DNA 1 - 10 ng bacterial DNA 0.1 - 1 ng plasmid DNA
<b>Final volume (Mix 1)</b>	<b>25 µl</b>	

<sup>a)</sup> To prepare Master Mix 1 for more than one reaction, multiply the amounts of all components of Master Mix 1 (except template DNA) by Z, where Z = the number of reactions to be run + one additional reaction. Mix these components to form a homogeneous solution, aliquot equal amounts of the solution to new 1.5 ml microfuge tubes (one for each reaction, on ice), add 100 - 500 ng template DNA to each, and label each new tube "Master Mix 1 + (template name)".

#### Master Mix 2 (for one reaction)

Components (Mix 2)	Volume <sup>b</sup>	Final conc.
PCR Grade water	18.75 µl	
Taq PCR buffer + MgCl <sub>2</sub> , 10x conc.	5 µl	1.5 mM MgCl <sub>2</sub>
Taq DNA Polymerase, 1 U/µl	1.25 µl	1.25 U/50 µl reaction
<b>Final volume (Mix 1)</b>	<b>25 µl</b>	

<sup>b)</sup> To prepare Master Mix 2 for more than one reaction, multiply the amounts of all ingredients by Z, where Z = the number of reactions to be run + one additional reaction.

- 3 Mix the reagents in each tube and centrifuge briefly.
- 4 ▶ For each reaction, combine 25 µl Mix 1 (including DNA template) and 25 µl Mix 2 in a thin-walled PCR tube (or well of a PCR microplate) on ice.  
▶ Gently mix the solutions in each PCR tube (or well) to produce a homogeneous reaction mixture.  
▶ According to the instructions supplied with your instrument, prepare the tubes or microplate for PCR (e.g., overlay reaction mixture in tube with mineral oil or seal the microplate with adhesive foil).
- 5 Place PCR tubes (or microplate) in the PCR instrument and begin PCR immediately.  
 The completed reaction mixtures should not be stored for extended periods of time.

4

## PCR

After placing the samples in the PCR instrument, cycle according to the thermal profile below.



The thermal profile below was developed for the Applied Biosystems GeneAmp PCR System 2400 thermal block cycler. Other PCR instruments may require a different profile.

Cycles		Temperature	Time
<b>Initial Denaturation</b>			
1		94°C	2 min
<b>Amplification</b>			
25 – 30	Denaturation	94°C	15 – 30 s
	Annealing	50 to 65°C <sup>a</sup>	30 – 60 s
	Elongation	72°C	45 s – 2 min <sup>b</sup>
<b>Final Elongation</b>			
1		72°C	7 min

<sup>a)</sup> Optimal annealing temperature depends on the melting temperature of the primers and the system used.

<sup>b)</sup> Elongation time depends on fragment length. Use 45 s for targets that are 1 kb or shorter, 1 min for 1.0 – 1.5 kb targets, 2 min for 1.5 – 3 kb targets.

## Troubleshooting the Amplification

### Optimizing Reaction Conditions

For initial experiments, use the conditions listed in the protocols above, which generally give good results. However, if initial results are unsatisfactory, use the following guidelines to optimize the critical components of the reaction:

Parameter	Optimal Concentration or Condition
Template DNA	Ideally, the sample should contain at least 10 <sup>5</sup> – 10 <sup>6</sup> target molecules. However, for complex genomic DNA (e.g. human genomic DNA), this will not be possible.  In general, never use less than 50 ng or more than 200 ng complex DNA in any reaction that uses Taq DNA Polymerase. To amplify higher amounts of DNA, try using a hot start enzyme. (See section 4.1.1.)
Taq DNA Polymerase	between 1 and 2.5 U per 50 µl reaction
Primers	between 100 and 600 nM (final concentration of each primer)
MgCl <sub>2</sub>	between 1 and 5 mM <sup>b</sup>

<sup>a)</sup> If you are amplifying single copy genes, then, on average, there are 3 × 10<sup>5</sup> target molecules in:

- ▶ 1 µg human genomic DNA
- ▶ 10 ng yeast DNA
- ▶ 1 ng *E. coli* DNA

<sup>b)</sup> For optimization, use the PCR reaction buffer without MgCl<sub>2</sub> and the 25 mM MgCl<sub>2</sub> solution (supplied with enzyme) to produce the following concentrations:



**If you add the following volume of 25 mM MgCl<sub>2</sub> stock to a 50 µl (final volume) PCR mixture:**

2 µl	2.5 µl	3 µl	3.5 µl	4 µl	5 µl	10 µl
------	--------	------	--------	------	------	-------

**You will end up with a final MgCl<sub>2</sub> reaction concentration of:**

1 mM	1.25 mM	1.5 mM	1.75 mM	2 mM	2.5 mM	5 mM
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*Optimal reaction conditions (reagent concentrations, incubation times and temperatures, etc.) vary from system to system. For general information on optimizing a PCR reaction, see section 4.7, “Guidelines for Optimizing PCR”.*

### More Troubleshooting Options

For additional troubleshooting options, see “Troubleshooting” in the Appendix.

### Processing of PCR Products

#### Analysis

Analyze the products on a TBE buffered 1–2% agarose gel (dependant on the length of PCR products).

#### Cloning

We recommend TA cloning for direct cloning of PCR products generated with Taq DNA Polymerase as they usually have a single A overhang (3' end).



*To facilitate direct cloning of amplified DNA, restriction enzyme digestion may be performed directly in the PCR mix after amplification (i.e., without first purifying the PCR products). For this to work, the restriction enzyme must show good activity in the reaction buffer for Taq DNA Polymerase. To develop guidelines for which restriction enzymes are suitable for this application, our laboratories tested many of our restriction enzymes for activity in Taq DNA Polymerase buffer (Blanck et al., 1997; Blanck et al., 1999). Several restriction enzymes were found to have 100% of their normal activity in this buffer and therefore would be suitable for direct digestion of PCR products after amplification. For a list of these enzymes, see Chapter 6.*

## 4.2 High Fidelity PCR

### Purpose of Procedure:

Extremely accurate amplification of normal DNA templates up to 5 kb in length

### Enzyme/System Needed:

Pwo SuperYield DNA Polymerase (for templates up to 3 kb), or

FastStart High Fidelity System (for templates up to 5 kb), or

Expand High Fidelity PCR System (for templates up to 5 kb)



*For more information on choosing the correct amplification procedure, see the PCR Protocol Selection Guide at the beginning of Chapter 4.*

### 4.2.1 Reagents and Equipment Required

#### PCR Enzyme or System Required

The protocols given in section 4.2.3 below require high fidelity enzymes and systems with proofreading activity as well as polymerase activity, to ensure highly accurate transcription of DNA targets:

- ▶ For amplification of targets up to 3 kb long and available in limited quantity: Use Pwo SuperYield DNA Polymerase.
- ▶ For amplification of targets up to 5 kb long: Use the FastStart High Fidelity PCR System or the Expand High Fidelity PCR System



*FastStart High Fidelity PCR System is an ideal tool for carryover prevention (see Chapter 2).*

*The FastStart High Fidelity PCR System may also be used to amplify mixtures of targets (multiplexing). For details, see Chapter 8.*

*You can also perform high fidelity PCR with the High Fidelity PCR Master, a ready-to-use, 2x reaction mixture (containing high fidelity enzyme blend, buffer, nucleotides and MgCl<sub>2</sub>). This convenient product lets you prepare complete high fidelity PCR mixes with fewer pipetting steps. For a detailed high fidelity protocol that uses the FastStart PCR Master, see the package insert for catalog number 12 140 314 001, which is available online at [www.roche-applied-science.com](http://www.roche-applied-science.com).*

*For details on the characteristics of the High Fidelity Enzymes and Systems, see Chapter 2. For pack sizes, see Ordering Information in the Appendix.*

# 4

### Additional Equipment and Reagents Required

- ▶ PCR Grade Nucleotide Mix\* (10 mM of each dNTP)



*The PCR Grade Nucleotide Mix is available either separately or as part of a convenient enzyme-dNTPack (e.g., Pwo SuperYield DNA Polymerase, dNTPack\*, FastStart High Fidelity PCR System, dNTPack\* or Expand High Fidelity PCR System, dNTPack\*)*

- ▶ PCR Grade Water \*
- ▶ Thermal block cycler (e.g., Applied Biosystems GeneAmp PCR System 2400) or other PCR instrument
- ▶ 0.2 ml thin-walled PCR tubes\* or PCR microplate, suitable for your PCR instrument
- ▶ Sterile reaction tubes for preparing master mixes and dilutions

\* available from Roche Applied Science; see Ordering Information (in the Appendix) for details.

## 4.2.2 General Considerations for High Fidelity PCR

### Sample Material

Any suitable DNA up to 5 kb in length, including abnormally GC-rich DNA. DNA samples should be as pure as possible.



*As with any PCR, the quality of the template influences the outcome of the PCR. For more information on preparing high quality DNA templates, see Chapter 3 of this manual.*

### Template Dilution Buffer

The optimal dilution buffer for template DNA is either sterile, double-distilled water or Tris (5–10 mM, pH 7–8).



*Do not dissolve the template in TE buffer because EDTA chelates Mg<sup>2+</sup>.*

### 4.2.3 Protocols for High Fidelity PCR

#### What We Mean by High Fidelity and Why It Is Important

For many applications that use PCR products, mutations introduced during PCR are of little concern. However, in some important applications (e.g., the amplification of genomic products for cloning or sequencing, the study of allelic polymorphisms and SNPs, or the characterization of rare mutations), any transcriptional errors that occur during the amplification reaction can lead to misleading results. Such errors can severely damage a research project and lead to huge costs (e.g., wasted money, loss of time, loss of reputation).

 Such transcriptional errors can also be devastating if the target DNA is only available in very limited quantities or if the target DNA is a complex mixture (e.g., for multiplexing).

To avoid such costly errors, the target DNA must be transcribed with very high fidelity. That is, there needs to be a proofreading mechanism that can correct errors (incorporation of incorrect bases) during transcription, so the final product containing no (or only a few) mistakes.

The easiest way to ensure high fidelity transcription is to use a proofreading polymerase. Such a polymerase has a 3'-5' exonuclease activity that can remove misincorporated nucleotides from the transcript during the polymerase reaction.

For high fidelity transcription, we do not recommend using Taq DNA Polymerase alone, since Taq DNA Polymerase does not have such a proofreading activity. A better solution is to use a true proofreading polymerase (e.g., Pwo SuperYield DNA Polymerase) that has the required proofreading activity. Pwo SuperYield DNA Polymerase has an 18-fold lower transcriptional error rate than Taq DNA Polymerase.

 For details on how the transcriptional error rate is determined, see the literature (e.g., Barnes, 1994).

Unfortunately, Pwo SuperYield DNA Polymerase can only amplify relative short (up to 3 kb) DNA targets. For high fidelity transcription of longer targets, we recommend using an enzyme blend (e.g., the FastStart High Fidelity PCR System or the Expand High Fidelity PCR System), which combines the high processivity of Taq DNA Polymerase with a proofreading protein to correct transcriptional errors.

The High Fidelity PCR protocols described in this section are designed for applications where transcriptional accuracy is vital to the success of the experiment.

#### Protocol D: High Fidelity Amplification of up to 3 kb DNA with Pwo SuperYield Polymerase

##### About Pwo SuperYield DNA Polymerase

Pwo SuperYield DNA Polymerase has both a polymerase activity and a 3'-5' exonuclease (proofreading) activity. Because of this proofreading activity, the polymerase transcribes template DNA approximately 18-fold more accurately than Taq DNA Polymerase.



## Designing Primers

The proofreading activity of Pwo SuperYield DNA Polymerase will cleave single-stranded DNA (e.g., PCR primers). You should therefore take this activity into account when designing primers. To ensure that the first 15 bases at the 5' end of each primer are protected from degradation, make each primer 20–35 bases long. The 3' end of each primer should be as homologous to the binding site as possible.

 For additional resistance to slow degradation, consider incorporating nuclease-resistant dNTPs (e.g., phosphorothionate nucleotides) into the primers. Alternatively, maximize the GC content of the primers.

## Setting Up the Reaction

- 1  Thaw all frozen reagents before use.  
 Mix all reagents and briefly centrifuge them before starting the procedure.
- 2 Prepare the master mix in a sterile 1.5 ml reaction tube (on ice).  
 Add the components in the order listed below. Numbered vials are supplied with the enzyme.

### Master Mix (for one reaction) <sup>a</sup>

Components	Volume	Final conc.
PCR Grade water	variable	to make final volume (including template) = 50 µl
Pwo SuperYield PCR Buffer with MgCl <sub>2</sub> , 10× conc. (vial 2)	5 µl	1.5 mM MgCl <sub>2</sub>
PCR Grade Nucleotide Mix, 10 mM of each	1 µl	200 µM (each nucleotide)
Downstream primer	variable	200 - 300 nM
Upstream primer	same as downstream primer	200 - 300 nM
GC-RICH Resolution Solution, 5× conc. (vial 3; optional) <sup>b</sup>	10 µl	1×
Pwo SuperYield DNA Polymerase (vial 1)	0.5 µl	2.5 U per reaction
 Mix all the above ingredients thoroughly and transfer the whole mix to a PCR tube or well of a microplate. If you prepared Master Mix for more than one reaction <sup>a</sup> , pipet equal volumes of it into separate PCR tubes (or wells).  Add template DNA to the individual tubes (or wells) containing Master Mix, using the following guidelines:		
DNA template	variable	50–200 ng complex DNA 10 pg–100 ng plasmid DNA
<b>Final volume (including template)</b>	<b>50 µl</b>	1× Reaction Mix

<sup>a</sup>) To prepare a Master Mix for more than one reaction, multiply the amounts of all ingredients (except template) by Z, where Z = the number of reactions to be run + one additional reaction.

<sup>b</sup>) Use GC-RICH Resolution Solution only if template has an abnormal GC content or sequences that are difficult to amplify. The solution is not required for amplification of DNA with normal GC content.



# 4

- 3 ▶ Gently mix the solutions in each PCR tube (or well) to produce a homogeneous reaction mixture.
  - ▶ According to the instructions supplied with your instrument, prepare the tubes or microplate for PCR (e.g., overlay reaction mixture in tube with mineral oil or seal the microplate with adhesive foil).
- 4 Place PCR tubes (or microplate) in the PCR instrument and begin PCR immediately.
  - ! *The completed reaction mixtures should not be stored for extended periods of time.*

## PCR

After placing the samples in the PCR instrument, cycle according to the thermal profile below.

! *The thermal profile below was developed for the Applied Biosystems GeneAmp PCR System 2400, 2700, and 9600 thermal block cyclers. Other PCR instruments may require a different profile.*

Cycles		Temperature	Time
<b>Initial Denaturation</b>			
1		92 to 95°C <sup>a</sup>	2 min
<b>Amplification</b>			
30 <sup>b</sup>	Denaturation	92 to 95°C <sup>a</sup>	15 s
	Annealing	44 to 65°C <sup>c</sup>	30 s
	Elongation	72°C	0.5–3 min <sup>d</sup>
<b>Final Elongation</b>			
1		72°C	up to 7 min

- <sup>a)</sup> Denaturation temperature depends on the template. Always use the lowest temperature that will completely denature the template. Higher temperatures may lead to depurination of the template DNA and reduced product yield.
- <sup>b)</sup> Number of cycles required depends on initial amount of template. For low abundance targets, use more cycles.
- <sup>c)</sup> Optimal annealing temperature depends on the melting temperature of the primers and the system used.
- <sup>d)</sup> Elongation time depends on fragment length. Use 1 min/kb for targets up to 3 kb long.

## Protocol E: High Fidelity Amplification of up to 5 kb DNA with FastStart High Fidelity PCR System

### FastStart High Fidelity PCR System

The FastStart High Fidelity PCR System is an innovative, novel blend that is optimized for specificity, sensitivity and yield. The blend contains two proteins:

- ▶ Chemically modified FastStart Taq DNA Polymerase, the activity of which is activated by a 2 min incubation at 95°C.
- ▶ A chemically modified, thermostable proofreading protein that has no polymerase activity.

4

This enzyme blend is inactive at temperatures below 75°C and therefore will not elongate nonspecific primer-template hybrids that may form during reaction set-up. Once activated, FastStart Taq DNA Polymerase is highly processive. In addition, the proof-reading protein excises incorrectly incorporated nucleotides to ensure accurate transcription. This unique combination can transcribe DNA (up to 5 kb) approximately fourfold more accurately than Taq DNA Polymerase alone.

### Setting Up the Reaction

- 1 ▶ Thaw all frozen reagents before use.  
 ▶ Mix all reagents and briefly centrifuge them before starting the procedure.
- 2 Prepare the master mix in a sterile 1.5 ml reaction tube (on ice).  
 *Add the components in the order listed below. Numbered vials are supplied with the enzyme.*

#### Master Mix (for one reaction)<sup>a</sup>

Components	Volume	Final conc.
PCR Grade water	variable	to make final volume (including template) = 50 µl
FastStart High Fidelity Reaction Buffer with MgCl <sub>2</sub> , 10× conc. (vial 2)	5 µl	1.8 mM MgCl <sub>2</sub>
DMSO, 100% (vial 5; optional) <sup>b</sup>	variable, up to 5 µl	up to 10%
PCR Grade Nucleotide Mix, 10 mM of each	1 µl	200 µM (each nucleotide)
Downstream primer	variable	400 nM
Upstream primer	same as downstream primer	400 nM
FastStart High Fidelity Enzyme Blend (vial 1)	0.5 µl	2.5 U per reaction
▶ Mix all the above ingredients thoroughly and transfer the whole mix to a PCR tube or well of a microplate.  <i>If you prepared Master Mix for more than one reaction<sup>a</sup>, pipet equal volumes of it into separate PCR tubes (or wells).</i> ▶ Add template DNA to the individual tubes (or wells) containing Master Mix, using the following guidelines:		
DNA template	variable	5–250 ng complex DNA 0.1–10 ng plasmid DNA
<b>Final volume (including template)</b>	<b>50 µl</b>	<b>1× Reaction Mix</b>

<sup>a)</sup> To prepare a Master Mix for more than one reaction, multiply the amounts of all ingredients (except template) by Z, where Z = the number of reactions to be run + one additional reaction.

<sup>b)</sup> Use DMSO only if template has an abnormal GC content or sequences that are difficult to amplify. The solution is not required for amplification of DNA with normal GC content.

- 3 ▶ Gently mix the solutions in each PCR tube (or well) to produce a homogeneous reaction mixture.  
 ▶ According to the instructions supplied with your instrument, prepare the tubes or microplate for PCR (e.g., overlay reaction mixture in tube with mineral oil or seal the microplate with adhesive foil).
- 4 Place PCR tubes (or microplate) in the PCR instrument and begin PCR immediately.  
 *The completed reaction mixtures should not be stored for extended periods of time.*

4

## PCR

After placing the samples in the PCR instrument, cycle according to one of the thermal profiles below.

 *The thermal profiles below were developed for the Applied Biosystems GeneAmp PCR System 2400 thermal block cycler. Other PCR instruments may require a different profile.*

Thermal Profile A (for fragments up to 3 kb long):

Cycles		Temperature	Time
<b>Initial Denaturation</b>			
1		95°C	2 min
<b>Amplification</b>			
30 <sup>b</sup>	Denaturation	95°C	30 s
	Annealing	55 to 72°C <sup>b</sup>	30 s
	Elongation	72°C	0.5–3 min <sup>c</sup>
<b>Final Elongation</b>			
1		72°C	4–7 min

Thermal Profile B (for 3 – 5 kb fragments):

Cycles		Temperature	Time
<b>Initial Denaturation</b>			
1		95°C	2 min
<b>Amplification Program 1</b>			
30 <sup>b</sup>	Denaturation	95°C	10–30 s
	Annealing	55 to 68°C <sup>b</sup>	30 s
	Elongation	68°C	3–5 min <sup>c</sup>
<b>Amplification Program 2</b>			
30 <sup>b</sup>	Denaturation	95°C	10–30 s
	Annealing	55 to 68°C <sup>b</sup>	30 s
	Elongation	68°C	3–5 min <sup>c</sup> + 20 s cycle elongation for each successive cycle <sup>e</sup>
<b>Final Elongation</b>			
1		72°C	7 min

- <sup>a)</sup> This step serves two purposes. It activates the FastStart Taq DNA Polymerase as well as denaturing the template.
- <sup>b)</sup> Optimal annealing temperature depends on the melting temperature of the primers and the system used.
- <sup>c)</sup> Elongation time depends on fragment length. Use 1 min/kb for amplicons up to 5 kb long.
- <sup>d)</sup> Number of cycles required depends on initial amount of template. For low abundance targets or complex target mixtures (multiplexing), use more cycles.
- <sup>e)</sup> Gradually increasing extension time ensures a higher yield of amplification products. For example, cycle no. 11 is 20 s longer than cycle 10, cycle no. 12 is 40 s longer than cycle 10, cycle no. 13 is 60 s longer than cycle 10, etc.

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## Protocol F: High Fidelity Amplification of up to 5 kb DNA with Expand High Fidelity PCR System

### About the Expand High Fidelity PCR System

The Expand High Fidelity PCR System is a mixture of two proteins:

- ▶ Thermostable Taq DNA Polymerase
- ▶ Thermostable DNA polymerase with proofreading (3'-5' exonuclease) activity

Because of the proofreading activity of the second polymerase, this enzyme mix transcribes template DNA approximately threefold more accurately than Taq DNA Polymerase alone.

 *The Expand High Fidelity PCR System is optimized for high fidelity amplification of fragments up to 5 kb long. However, it will also amplify longer fragments; however the product yield will decrease as the fragment length increases.*

### Designing Primers

Primer design is very critical for successful amplification of long targets. At the very least, design primer sequences that have minimal potential to form secondary structure or dimers.

#### Setting Up the Reaction

- 1 ▶ Thaw all frozen reagents before use.  
 ▶ Mix all reagents thoroughly and briefly centrifuge them before starting the procedure.
- 2 Prepare two separate master mixes in sterile 1.5 ml microfuge tubes (on ice). (Master Mix 1 contains dNTPs, primers, and template DNA; Master Mix 2 contains PCR reaction buffer and enzyme mix.)  
 *For each mix, add the components in the order listed below.*

#### Master Mix 1 (for one reaction)

Components (Mix 1)	Volume <sup>a</sup>	Final conc.
PCR Grade water	variable	to make final volume (including template) = 25 µl
PCR Grade Nucleotide Mix, 10 mM of each	1 µl	200 µM (each nucleotide)
Downstream primer	variable	300 nM
Upstream primer	same as downstream primer	300 nM
Template DNA	variable	10–250 ng complex DNA 0.1–15 ng plasmid DNA
<b>Final volume (including template)</b>	<b>25 µl</b>	

<sup>a)</sup> To prepare Master Mix 1 for more than one reaction, multiply the amounts of all components of Master Mix 1 (except template DNA) by Z, where Z = the number of reactions to be run + one additional reaction. Mix these components to form a homogeneous solution, aliquot equal amounts of the solution to new 1.5 ml microfuge tubes (one for each reaction, on ice), add 0.1 – 250 ng template DNA to each, and label each new tube “Master Mix 1 + (template name)”.



4

<b>Master Mix 2 (for one reaction)</b>		
<b>Components (Mix 2)</b>	<b>Volume <sup>b</sup></b>	<b>Final conc.</b>
PCR Grade water	19.25 µl	
Expand High Fidelity buffer with MgCl <sub>2</sub> , 10× conc. (vial 2)	5 µl	1.5 mM MgCl <sub>2</sub>
Expand High Fidelity enzyme mix (vial 1)	0.75 µl	2.6 U/50 µl reaction
<b>Final volume (Mix 2)</b>	<b>25 µl</b>	

<sup>b)</sup> To prepare Master Mix 2 for more than one reaction, multiply the amounts of all ingredients by Z, where Z = the number of reactions to be run + one additional reaction.

- 3** Mix the reagents in each tube and centrifuge briefly.
- 4**
  - ▶ For each reaction, combine 25 µl Mix 1 (including template) and 25 µl Mix 2 in a thin-walled PCR tube (or well of a PCR microplate) on ice.
  - ▶ Gently mix the solutions to produce a homogeneous reaction mixture.
  - ▶ According to the instructions supplied with your instrument, prepare the tubes or microplate for PCR (e.g., overlay reaction mixture in tube with mineral oil or seal the microplate with adhesive foil).
- 5** Place PCR tubes in the PCR instrument and begin PCR immediately.
  -  *The completed reaction mixtures should not be stored for extended periods of time.*



### PCR

After placing the samples in the PCR instrument, cycle according to the thermal profile below.

-  *The thermal profile below was developed for the Applied Biosystems GeneAmp PCR System 2400 thermal block cycler. Other PCR instruments may require a different profile.*

Cycles		Temperature	Time
<b>Initial Denaturation</b>			
1		94°C	2 min
<b>Amplification Program 1</b>			
30 <sup>b</sup>	Denaturation	94°C	15 s
	Annealing	45 to 65°C <sup>a</sup>	30 s
	Elongation	72 or 68°C <sup>b</sup>	0.75 - 8 min <sup>c</sup>
<b>Amplification Program 2</b>			
30 <sup>b</sup>	Denaturation	94°C	15 s
	Annealing	45 to 65°C <sup>a</sup>	30 s
	Elongation	72 or 68°C <sup>b</sup>	0.75 - 8 min <sup>c</sup> + 5 s cycle elongation for each successive cycle <sup>e</sup>
<b>Final Elongation</b>			
1		72°C	7 min

- <sup>a)</sup> Optimal annealing temperature depends on the melting temperature of the primers and the system used.
- <sup>b)</sup> Use 72°C for targets up to 3 kb. For targets >3 kb, use 68°C.
- <sup>c)</sup> Elongation time depends on fragment length. Use 45 s for targets up to 0.75 kb, 1 min for 1.5 kb, 2 min for 3 kb, 4 min for 6 kb, 8 min for up to 9 kb.
- <sup>d)</sup> Number of cycles required depends on initial amount of template. For low abundance targets, use more cycles.
- <sup>e)</sup> Gradually increasing extension time ensures a higher yield of amplification products. For example, cycle no. 11 is 5 s longer than cycle 10, cycle no. 12 is 10 s longer than cycle 10, cycle no. 13 is 15 s longer than cycle 10, etc.

# 4

## Troubleshooting the Amplification

### Optimizing Reaction Conditions

For initial experiments, use the conditions listed in the protocols above, which generally give good results. However, if initial results are unsatisfactory, use the following guidelines to optimize the critical components of the reaction:

Parameter	Optimal Concentration or Condition
Template DNA	Protocol D (up to 3 kb; section C.3.1): 50–200 ng complex DNA (e.g., human genomic DNA) or 10 pg–100 ng plasmid DNA Protocol E (up to 5 kb; section C.3.2): 5–250 ng complex DNA (e.g., human genomic DNA) or 100 pg–10 ng plasmid DNA Protocol F (up to 5 kb; section C.3.3): 10–250 ng complex DNA (e.g., human genomic DNA) or 100 pg–15 ng plasmid DNA
Primers	Protocol D: 200–300 nM (final concentration of each primer) Protocol E: 200–600 nM (final concentration of each primer) Protocol F: No optimization necessary. Use the concentration (300 nM) recommended in the protocol.
PCR Enzyme	Protocol D: 0.5–5.0 U Pwo SuperYield DNA Polymerase per 50 µl reaction Protocol E: 0.5–5.0 U FastStart High Fidelity Enzyme Blend per 50 µl reaction Protocol F: No optimization necessary. Use the amount of Expand High Fidelity Enzyme Mix (2.6 U/rx) recommended in the protocol.
MgCl <sub>2</sub>	Protocol D: No optimization necessary. Use the concentration (1.5 mM) recommended in the protocol. Protocol E: 1.4–4.0 mM <sup>a</sup> Protocol F: 1.5–4.0 mM <sup>a</sup>

<sup>a)</sup> For optimization, use the PCR reaction buffer without MgCl<sub>2</sub> (supplied with enzyme) and titrate the Mg<sup>2+</sup> concentration in 0.5 mM steps. Use the 25 mM MgCl<sub>2</sub> solution (supplied with enzyme) to produce the following concentrations:

#### If you add the following volume of 25 mM MgCl<sub>2</sub> stock to a 50 µl (final volume) PCR mixture:

2 µl      3 µl      4 µl      5 µl      6 µl      7 µl      8 µl

#### You will end up with a final MgCl<sub>2</sub> reaction concentration of:

1 mM      1.5 mM      2 mM      2.5 mM      3 mM      3.5 mM      4 mM



*Optimal reaction conditions (reagent concentrations, incubation times and temperatures, etc.) vary from system to system. For general information on optimizing a PCR reaction, see section 4.7, “Guidelines for Optimizing PCR.”*

### More Troubleshooting Options

For additional troubleshooting options, see “Troubleshooting” in the Appendix.

### Processing of PCR Products

#### Storage

PCR products up to 5 kb long may be stored frozen for later use.



### Analysis

Analyze the products on a 0.6—2% agarose gel. Use TAE or TBE buffer, the percentage of the gel and the buffer system depend on the length of PCR fragments.

### Cloning

PCR products generated with Pwo SuperYield DNA Polymerase (Protocol D) have blunt ends. They can be used directly for blunt-end cloning.



*To facilitate direct cloning of amplified DNA, restriction enzyme digestion may be performed directly in the PCR mix after amplification (i.e., without first purifying the PCR products). For this to work, the restriction enzyme must show good activity in the reaction buffer for Pwo SuperYield DNA Polymerase. To develop guidelines for which restriction enzymes are suitable for this application, our laboratories tested many of our restriction enzymes for activity in Pwo SuperYield DNA Polymerase buffer (Meurer et al, 2005). Several restriction enzymes (e.g., Dpn I) were found to have 100% of their normal activity in this buffer and therefore would be suitable for direct digestion of PCR products after amplification. For a list of these enzymes, see Chapter 6.*

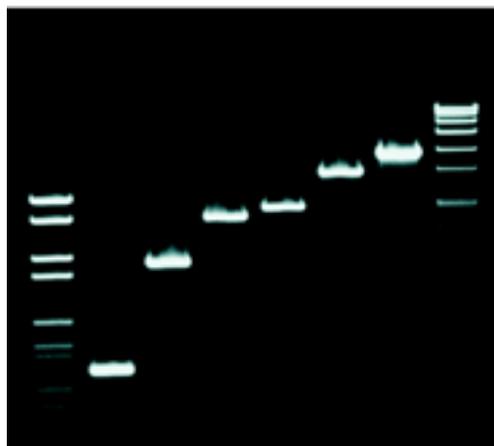
PCR products generated with the FastStart High Fidelity PCR System (Protocol E) or the Expand High Fidelity PCR System (Protocol F) usually have a single A overhang (3' end). We recommend TA cloning.

# 4

## 4.2.4 Typical Results

**Result:** Pwo SuperYield DNA Polymerase can amplify up to 3 kb fragments (from human genomic DNA) and up to 4 kb (from plasmid DNA) with excellent yield and specificity.

1 2 3 4 5 6 7 8

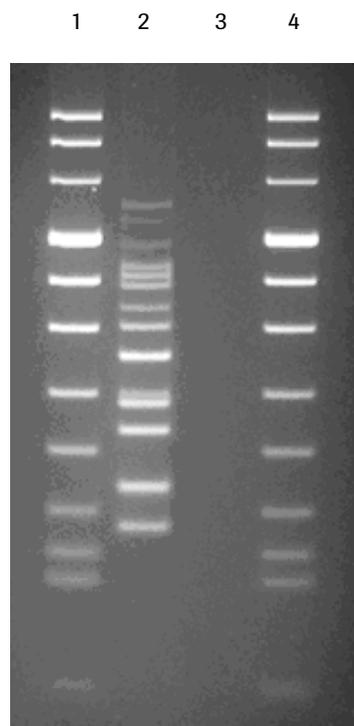


**Figure 4.2.1. Amplification of fragments of various lengths with Pwo SuperYield DNA Polymerase.** Fragments were amplified from DNA obtained from different sources. In all cases, Protocol A (above) was followed, but the reaction mixes did not contain GC-RICH Resolution Solution.

The length, amount and source of the DNA used for each experiment were:

- Lanes 1, 8:** DNA Molecular Weight Markers VI, VII, respectively
- Lane 2:** 0.3 kb tPA fragment from 200 ng human genomic DNA
- Lane 3:** 1.1 kb collagen fragment from 200 ng human genomic DNA
- Lane 4:** 1.7 kb tPA fragment from 200 ng human genomic DNA
- Lane 5:** 1.9 kb fragment from 200 ng potato DNA
- Lane 6:** 2.9 kb p53 fragment from 200 ng human genomic DNA
- Lane 7:** 3.6 kb fragment from 5 ng plasmid DNA

**Result:** The FastStart High Fidelity PCR System amplifies all fragments, producing a clear and specific banding pattern.



**Figure 4.2.2. Multiplex PCR with the FastStart High Fidelity PCR System.** A 14-plex PCR with 28 primers was performed to detect selected parts of the CFTR (Cystic Fibrosis Transmembrane Conductance Regulatory) gene. The amplicon length varies from 198 to 598 bp.

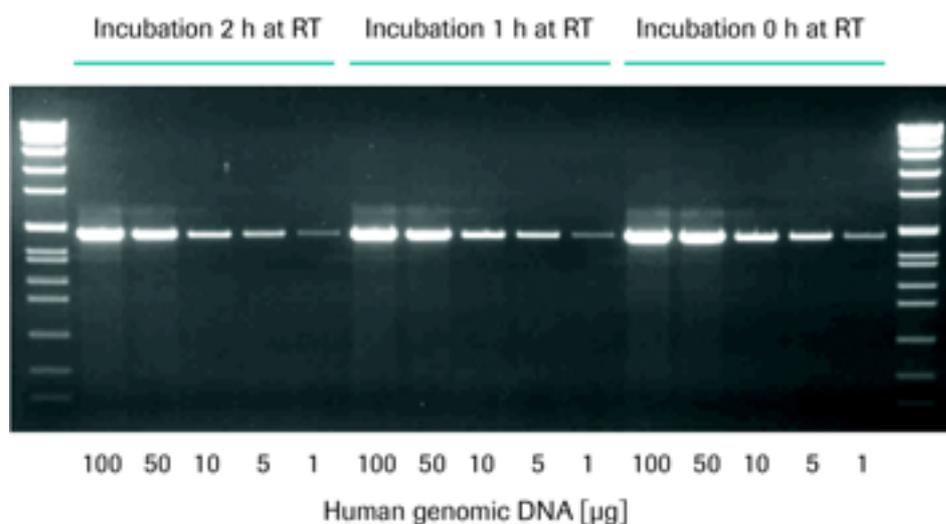
**Lanes 1 and 4:** DNA Molecular Weight Marker VIII (19 – 1114 bp)

**Lane 2:** Amplification (in the same reaction) of 14 different fragments (198 to 598 bp) from 100 ng human genomic DNA

**Lane 3:** Same reaction as in lane 2, but without template (negative control)

4

**Result:** The EPO target was specifically amplified with good yield in all tubes. No differences were seen between the three sets of reaction tubes, indicating that the total FastStart High Fidelity reaction mix (containing all reaction components) was stable for at least 2 h at RT. The exceptional room temperature stability of the entire reaction mix makes the FastStart High Fidelity reaction mix the product of choice when high throughput experiments require reactions to be set up automatically by robotic pipetting devices.



**Figure 4.2.3. Stability of FastStart High Fidelity reaction mix.** Varying amounts of human genomic DNA were used as template in a PCR with the FastStart High Fidelity reaction mix. The total reaction mix was pipetted into each of three sets of tubes at room temperature (RT). One set of tubes was placed in a thermocycler without delay and PCR was started immediately. The other two sets were left at RT for either 1 h or 2 h before PCR was started. In all cases, the product amplified was a 1.8 kb fragment from the erythropoietin (EPO) gene.

## 4.3 Long Template PCR

### Purpose of Method:

Amplification of 5–35 kb genomic DNA targets

### Enzyme/System Needed:

Expand Long Range dNTPack Kit (for 5–25 kb targets), or

Expand 20 kb<sup>PLUS</sup> PCR System (for 20–35 kb targets)



*For more information on choosing the correct amplification procedure, see the PCR Protocol Selection Guide at the beginning of Chapter 4.*

### 4.3.1 Reagents and Equipment Required

#### Kit Required

The protocols given in section 4.3.3 below require one of the following kits:

- ▶ For amplification of genomic DNA targets that are 5–25 kb long: Use the **Expand Long Range dNTPack Kit**.
- ▶ For amplification of genomic DNA targets that are 20–35 kb long: Use the **Expand 20 kb<sup>PLUS</sup> PCR System**.



*For details on the characteristics of the Expand PCR Systems, see Chapter 2. For pack sizes, see Ordering Information in the Appendix.*

#### Additional Reagents and Equipment Required

- ▶ PCR Grade Nucleotide Mix\* (10 mM of each dNTP)



*The PCR Grade Nucleotide Mix is available either separately or as part of a convenient enzyme-dNTPack (e.g., Expand Long Range dNTPack Kit\* or Expand 20 kb<sup>PLUS</sup> PCR System, dNTPack\*)*

- ▶ PCR Grade Water \*
- ▶ Thermal block cycler (e.g., Applied Biosystems GeneAmp PCR System 2400) or other PCR instrument
- ▶ 0.2 ml thin-walled PCR tubes\* or PCR microplate, suitable for your PCR instrument
- ▶ Sterile 1.5 ml microfuge tubes for preparing master mixes and dilutions

\* available from Roche Applied Science; see Ordering Information (in the Appendix) for details.

## 4.3.2 General Considerations for Long Template PCR

### Sample Material

Template DNA (e.g., long human genomic DNA fragments)



*The quality of the template has a tremendous effect on the success of the PCR. For example, if you are amplifying 20–35 kb targets, the average length of the DNA fragments in the sample should be >50 kb. Always check the length of the template DNA by agarose gel electrophoresis.*



*For more information on preparing high quality DNA templates, see Chapter 3.*

### Template Dilution Buffer

The optimal dilution buffer for template DNA is either sterile, double-distilled water or Tris (5–10 mM, pH 7–8).



*Do not dissolve the template in TE buffer because EDTA chelates  $Mg^{2+}$ .*

### Designing Primers

Primer design is very critical for successful amplification of long targets. Follow the following general guidelines:

- ▶ Primers should be between 22–34 nucleotides long
- ▶ The GC-content should be in the range of 45 to 65%
- ▶ Optimally there should be 2 G's or C's at the 3'-end of the primer
- ▶ Avoid high similarity (>80%) to other sequences in the genome
- ▶ Check primers for possible primer-dimer formation

If you have difficulties to amplify your sequence of interest, designing different primers or using different primer pairs may help to overcome the problem.

At the very least, you should design primer sequences that have minimal potential to form secondary structure and dimers.

### 4.3.3 Protocols for Long Template PCR

#### Protocol G: Amplification of 5–25 kb DNA with the Expand Long Range dNTPack

##### About the Expand Long Range dNTPack Kit

The Expand Long Range dNTPack Kit provides an easy, straightforward reaction set-up for long range PCR on 5 kb to 25 kb templates. Only one reaction buffer is needed, regardless of the size of the template. For convenience, we provide one reaction buffer with MgCl<sub>2</sub> and one buffer without MgCl<sub>2</sub>.

This second buffer could prove useful for those rare occasions when optimal results require adjustment of the Mg<sup>2+</sup> concentration. However, we have found that >95% of the PCR products tested in our labs can be obtained when the reaction buffer contains 2.5 mM MgCl<sub>2</sub> (the concentration in the first reaction buffer).

 *The DMSO concentration in the reaction should be adjusted, depending on the GC content of the template. You should determine the optimal DMSO concentration by a series of reactions in which the DMSO concentration is titrated (varied). Please remember that raising the DMSO concentration could also lower the annealing temperature.*

##### Setting Up the Reaction

-   Thaw 5× Buffer and DMSO 15 min at 37°.  
 Thaw all other reagents on ice.  
 Mix all reagents thoroughly and briefly centrifuge them before starting the procedure.  
 *Store all component on ice except of DMSO. Keep DMSO at room temperature.*



- 2 Prepare the Master Mix in a sterile microfuge tube on ice. Add the components in the order listed below.

**Master Mix** (for one reaction) <sup>a</sup>

Components	Volume	Final conc.
PCR Grade Water	Variable	to make final volume = 50 µl
PCR buffer with MgCl <sub>2</sub> , 5×	10 µl	1× buffer 2.5 mM MgCl <sub>2</sub>
PCR Nucleotide Mix, 10 mM	2.5 µl	500 µM
Downstream primer	Variable	300 nM
Upstream primer	same as downst- ream primer	300 nM
100% DMSO	Variable	0 – 10%, depending on the template
Template DNA	Variable	up to 500 ng genomic DNA <sup>b</sup>
Expand Long Range enzyme mix	0.7 µl	3.5 U/50 µl reaction
<b>Final volume</b>	<b>50 µl</b>	

<sup>a)</sup> To prepare a Master Mix for more than one reaction, multiply the amounts of all components in the mix by Z, where Z = the number of reactions to be run + one additional reaction.

<sup>b)</sup> For initial experiments, use 200 ng genomic DNA/50 µl reaction.

 For any amplification, the maximum amount of template DNA is 500 ng/50µl reaction. The yield of the reaction cannot be improved by using >500 ng genomic DNA; indeed, higher amounts of template DNA may inhibit the reaction.

- 3  Gently mix the solutions in each tube (or well) to produce a homogenous reaction mixture.  
 According to the instructions provided with your instrument, prepare the tubes or microplate for PCR.

- 4 Place PCR tubes (or microplate) in the PCR instrument and begin PCR immediately.  
 The completed reaction mixture should not be stored for extended periods of time.

### PCR

After placing the samples in the PCR instrument, cycle according to the thermal profile below.

 The protocol for the Expand Long Range dNTPack has been tested on the following PCR instruments:

-  Biometra: T3 Thermocycler
-  Eppendorf: Mastercycler Gradient
-  Applied Biosystems: GeneAmp PCR system 9700
-  BioRad: Tetrad 2



In our hands, the different thermal profiles and ramping rates of these thermal cyclers produced no significant differences in the results.

Cycles		Temperature	Time
<b>Initial Denaturation</b>			
1		92°C	2 min
<b>Amplification Program 1</b>			
10 <sup>b</sup>	Denaturation	92°C <sup>a</sup>	10 s <sup>a</sup>
	Annealing	45 to 65°C <sup>b</sup>	15 s
	Elongation	68°C	5–25 min <sup>c</sup>
<b>Amplification Program 2</b>			
20 <sup>b</sup>	Denaturation	92°C	15 s
	Annealing	45 to 65°C <sup>b</sup>	30 s
	Elongation	68°C	5–25 min <sup>c</sup> + 20 s cycle elongation for each successive cycle <sup>d</sup>
<b>Final Elongation</b>			
1		68°C	7 min

- <sup>a)</sup> In general, keep denaturation times as short as possible and denaturation temperature as low as possible.
- <sup>b)</sup> Optimal annealing temperature depends on the melting temperature of the primers and the system used.
- <sup>c)</sup> Elongation time depends on fragment length. Use 1 min elongation/kb length.
- <sup>d)</sup> Gradually increasing the extension time ensures a higher yield of amplification products. For example, cycle no. 11 is 20 s longer than cycle 10, cycle no. 12 is 40 s longer than cycle 10, cycle no. 13 is 60 s longer than cycle 10, etc.

### Protocol H: Amplification of 20–35 kb targets with the Expand 20 kb<sup>PLUS</sup> PCR System

#### Suitable Primers

Typical primers for amplification of 20–35 kb targets should be 22 – 34 nucleotides long and have similar melting temperatures >60°C.

Such long primers allow elongation to be performed at higher temperatures to enhance reaction specificity. This can be critical, since the amplification of long targets will be compromised if shorter nonspecific fragments are preferentially amplified.

 *Examples of suitable primers: The human β-globin forward primer (CAC AAG GGC TAC TGG TTG CCG ATT) and the reverse primer included in the Expand 20 kb<sup>PLUS</sup> PCR System kit can be used to amplify a 23 kb fragment from the human globin gene. The annealing temperature for these two primers is 62°C. These primers can be used in a control reaction to test the performance of other reaction components.*

Alternatively, if you use the forward primer TGC TGC TCT GTG CAT CCG AGT G with the human β-globin reverse primer included in the Expand 20 kb<sup>PLUS</sup> PCR System kit, and lower the annealing temperature to 60°C, you can amplify a 29.8 kb fragment from the human globin gene.



## Hot Start

Do not use AmpliWax. Instead, prepare two separate reaction mixes, then mix them together on ice just before starting PCR.

This strategy eliminates the need for a “Hot Start” technique, since the enzyme and the DNA (primers and template) are kept physically separate in the absence of dNTPs. Thus, the 3′–5′ exonuclease activity of the proofreading DNA polymerase (part of the Expand 20 kb<sup>PLUS</sup> enzyme mix) cannot partially degrade the primers and template.

### Setting Up the Reaction

- 1 ▶ Thaw all frozen reagents and place them on ice.  
 ▶ Mix all reagents briefly and centrifuge them before starting the procedure.
- 2 Prepare two separate master mixes. (Master Mix 1 contains dNTPs, primers, and template DNA; Master Mix 2 contains buffer and enzyme) in sterile microfuge tubes (on ice).

 For each mix, add the components in the order listed below.

#### Master Mix (for one reaction) <sup>a</sup>

Reagent	Volume	Final conc.
PCR Grade water	variable	
PCR Grade Nucleotide Mix, 10 mM	2.5 µl	500 µM (for each dNTP)
Downstream primer	variable <sup>b</sup>	400 nM
Upstream primer	same as downstream primer <sup>b</sup>	400 nM
Template DNA	variable <sup>b</sup>	250–500 ng genomic DNA
<b>Final volume (Mix 1)</b>	<b>25 µl</b>	

#### Master Mix 2 (for one reaction) <sup>a</sup>

Reagent	Volume	Final conc.
PCR Grade water	19 µl	
Expand 20 kb <sup>PLUS</sup> reaction buffer, 10×	5 µl	1× buffer 2.75 mM MgCl <sub>2</sub>
Expand 20 kb <sup>PLUS</sup> enzyme mix	1 µl	5 U/50 µl reaction
<b>Final volume (Mix 2)</b>	<b>25 µl</b>	

a) To prepare a Master Mix for more than one reaction, multiply the amounts of all components in the mix (except template DNA) by Z, where Z = the number of reactions to be run + one additional reaction.

b) If you use the control DNAs provided in the kit, then for the positive control sample:  
 downstream primer = 1 µl beta-globin control forward primer (vial 5 of kit)  
 upstream primer = 1 µl beta-globin control reverse primer (vial 6 of kit)  
 control template = 1.2 µl human genomic DNA (vial 4 of kit)

- 3 ▶ For each reaction, combine 25 µl Mix 1 (including template DNA) and 25 µl Mix 2 in a thin-walled PCR tube (or well of a PCR microplate) on ice.  
 ▶ Gently mix the solutions in each PCR tube (or well) to produce a homogeneous reaction mixture.  
 ▶ According to the instructions provided with your instrument, prepare the tubes or microplate for PCR.

- 4 Place PCR tubes (or microplate) in the PCR instrument and begin PCR immediately.  
 The completed reaction mixtures should not be stored for extended periods of time.

4

## PCR

After placing the samples in the PCR instrument, cycle according to the thermal profile below.



*The thermal profile below was developed for the Applied Biosystems GeneAmp PCR System 2400 thermal block cycler. Long template PCR is sensitive to even minute differences in ramping or heat transfer rates of different thermal block cyclers. Therefore, always develop and run your experiment on the same Applied Biosystems GeneAmp PCR System 2400 block cycler. If you switch to a different thermal block cycler or a different type of PCR instrument, adjust reaction conditions and thermal profile to ensure optimal results.*

Cycles		Temperature	Time
<b>Initial Denaturation</b>			
1		92°C	2 min
<b>Amplification Program 1</b>			
10	Denaturation	92°C	10 s
	Annealing	60 to 65°C <sup>a</sup>	30 s
	Elongation	68°C	11–30 min <sup>b</sup>
<b>Amplification Program 2</b>			
20–30 <sup>d</sup>	Denaturation	92 to 94°C	10 s
	Annealing	60 to 65°C <sup>a</sup>	30 s
	Elongation	68°C	11–30 min <sup>b</sup> + 10 s cycle elongation for each successive cycle <sup>c</sup>
<b>Final Elongation</b>			
1		68°C	7 min
<b>Cooling</b>			
1		4°C	unlimited time

<sup>a)</sup> Optimal annealing temperature depends on the melting temperature of the primers and the system used. Primers for this procedure should have annealing temperatures >60°C. For the control reaction, the annealing temperature is 62°C.

<sup>b)</sup> Elongation time depends on fragment length: For the control reaction, use 18 min. For other reactions, we recommend the following elongation times:

PCR fragment length (kb)	15	20	25	30	35	40	45
Elongation time (min)	11	14	17	20	23	27	30

 *Do not forget to extend the elongation time for each new cycle in the second part of the thermal cycling profile!*

- c) Gradually increasing the extension time ensures a higher yield of amplified products. For example, cycle no. 11 is 10 s longer than cycle 10, cycle no. 12 is 20 s longer than cycle 10, cycle no. 13 is 30 s longer than cycle 10, etc.
- d) The number of cycles depends on the amount of template (copies of target) DNA used. For human genomic DNA, we get good results with 250 ng of template and 30 cycles (total of amplification programs 1 and 2). However, increasing the total number of cycles to 35 or 40 may increase the yield of the amplicon.

## Troubleshooting the Amplification

### Optimizing Reaction Conditions

For initial experiments, use the concentrations and conditions listed in the protocols above, which generally good results. However, if initial results are unsatisfactory, use the following guidelines to optimize various reaction parameters:

Parameter	Optimal Concentration or Condition
Template DNA	Protocol G (5–25 kb targets; section 4.4.3.1): 50–500 ng genomic DNA Protocol H (20–35 kb targets; section 4.4.3.2): 250–500 ng genomic DNA  <i>See length-specific guidelines in Protocol A and Protocol B above.</i>
dNTPs	Always use a balanced concentration of dNTPs, <i>i.e.</i> , the same final concentration for each dNTP. For both protocols, the optimal concentration of dNTPs is 500 $\mu\text{M}$ (each dNTP).  <i>Do not arbitrarily change the dNTP concentrations listed in the protocols. However, if you do increase the concentration of dNTPs, always increase the concentration of <math>\text{Mg}^{2+}</math> ions in parallel.</i>
$\text{MgCl}_2$	Depends on enzyme mix used and dNTP concentration. For example, Protocol G uses 2.5 mM $\text{MgCl}_2$ for 500 $\mu\text{M}$ dNTPs, while Protocol H uses 2.75 mM $\text{MgCl}_2$ for 500 $\mu\text{M}$ dNTPs.  <i>Additional <math>\text{Mg}^{2+}</math> ions may increase the amplification yield. If you optimize nothing else, at least titrate the <math>\text{Mg}^{2+}</math> concentration.</i>

 *Optimal reaction conditions (reagent concentrations incubation times and temperatures, etc.) vary from system to system. For general information on optimizing a PCR reaction, see section 4.7, “Guidelines for Optimizing PCR”.*

### Optimizing Reaction Conditions

For additional troubleshooting options, see “Troubleshooting” in the Appendix of this manual.

## Processing of PCR Products

### Analysis

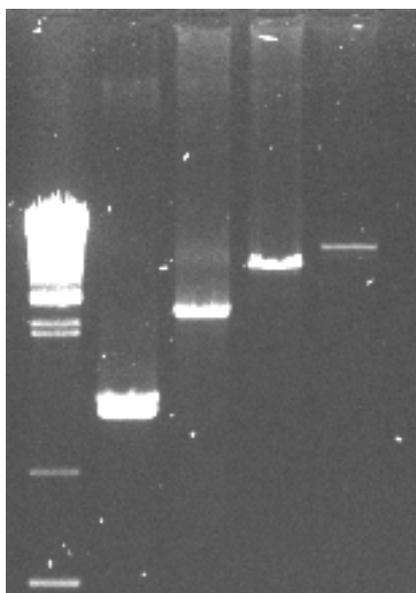
Analyze the products on a 0.6–1% agarose gel. Use TAE or TBE buffer, the percentage of the gel and the buffer system depend on the length of PCR fragments.

### Cloning

PCR products generated with the Expand Long Range or Expand 20 kb<sup>PLUS</sup> PCR System usually have a single A overhang (3' end). We recommend TA cloning.

### 4.3.4 Typical Results for Long Template PCR

1 2 3 4 5



**Figure 4.3.1. Amplification of long template DNA with Expand LongRange dNTPack.** The reaction mixes contained different amounts of DMSO as well as different sets of primers, which recognize various fragments of the tissue plasminogen activator (tPA) gene. Five  $\mu$ l of each PCR product was loaded onto a 0.75% agarose gel. The samples on the gel (from left to right) were generated with the following reaction components:

**Lane 1:** 300 ng Molecular Weight Marker XV\*.

**Lane 2:** 5.1 kb tPA fragment, amplified from 50 ng human genomic DNA in the presence of 1.5% DMSO.

**Lane 3:** 9.3 kb tPA fragment, amplified from 50 ng human genomic DNA in the presence of 6% DMSO.

**Lane 4:** 15 kb tPA fragment, amplified from 50 ng human genomic DNA in the presence of 6% DMSO.

**Lane 5:** 25.6 kb  $\beta$ -globin fragment, amplified from 200 ng human genomic DNA in the presence of 6% DMSO.

## 4.4 Amplification of Difficult Templates

### Purpose of Procedure:

Amplification of GC-rich targets up to 5 kb in length, DNA with repetitive sequences or mixtures of DNA with varying GC content.

### Enzyme/System Needed:

GC-RICH PCR System (for GC-rich templates up to 5 kb)

 *It may also be possible to amplify shorter GC-rich targets (up to 3 kb) with Pwo SuperYield DNA Polymerase (protocol D in section 4.2 of this chapter) or FastStart Taq DNA Polymerase plus GC-RICH Solution (protocol B in section 4.1 of this chapter).*

 *For more information on choosing the correct amplification procedure, see the PCR Protocol Selection Guide at the beginning of Chapter 4.*

### 4.4.1 Reagents and Equipment Required

#### Reagent Kit Required for these Protocols: GC-RICH PCR System

 *For details on the characteristics of the GC-RICH PCR System, see Chapter 2. For pack sizes, see Ordering Information in the Appendix.*

#### Additional Equipment and Reagents Required

- ▶ Thermal block cycler (e.g., Applied Biosystems GeneAmp PCR System 2400) or other PCR instrument
- ▶ 0.2 ml thin-walled PCR tubes\* or PCR microplate, suitable for your PCR instrument
- ▶ Sterile 1.5 ml microfuge tubes for preparing master mixes and dilutions
- ▶ PCR Grade Nucleotide Mix\* (10 mM of each nucleotide)

 *The PCR Grade Nucleotide Mix is available either separately or as part of a convenient enzyme-dNTPack (e.g., GC-RICH PCR System, dNTPack\*)*

\* available from Roche Applied Science; see Ordering Information (in the Appendix) for details.

## 4.4.2 General Considerations for Amplification of Difficult Templates

### Sample Material

Any suitable DNA up to 5 kb in length, including:

- ▶ GC-rich DNA
- ▶ DNA with repetitive sequences
- ▶ Mixture of DNAs with varying GC content (e.g., for multiplex PCR or construction of random fragment libraries)



*As with any PCR, the quality of the template influences the outcome of the PCR. For more information on preparing high quality DNA templates, see Chapter 3.*

### Template Dilution Buffer

The optimal dilution buffer for template DNA is either sterile, double-distilled water or Tris (5–10 mM, pH 7–8).



*Do not dissolve the template in TE buffer because EDTA chelates Mg<sup>2+</sup>.*

### Choice of Reaction Protocols

The GC-RICH PCR System includes three special components not included in most PCR systems:

- ▶ The special GC-RICH PCR enzyme mix (vial 1) and reaction buffer (vial 2) contain detergents and DMSO. Together, they are capable of amplifying many difficult templates.
- ▶ The special GC-RICH Resolution Solution (vial 3) is a reaction supplement that is specifically designed to amplify DNA with high GC content, including repetitive DNA sequences, or DNA mixtures that are not adequately amplified with GC-RICH reaction buffer alone.

Use the following table to determine which special component (and corresponding protocol) will give the best results for your template:

If amplifying...	Then use...
Mixture of DNAs that has varying GC content	Protocol I, which uses only the GC-RICH PCR enzyme mix and reaction buffer
DNA with a high GC content	Protocol K, which uses the GC-RICH PCR enzyme mix, reaction buffer, and resolution solution
Repetitive DNA	Protocol K
Mixture of DNAs that does not give the expected PCR yield or specificity with Protocol I	Protocol K

### 4.4.3 Protocols for Amplification of Difficult Templates (for Targets up to 5 kb)



It may also be possible to amplify shorter GC-rich targets (up to 3 kb) with Pwo SuperYield DNA Polymerase (protocol D in section 4.2 of this chapter) or FastStart Taq DNA Polymerase plus GC-RICH resolution solution (protocol B in section 4.1 of this chapter).

#### Protocol I: Amplification with GC-RICH PCR Enzyme Mix and Reaction Buffer

##### Setting Up the Reaction

- 1 ▶ Thaw all frozen reagents before use.  
▶ Mix all reagents thoroughly and briefly centrifuge them before starting the procedure.
- 2 Prepare two separate master mixes in sterile 1.5 ml microfuge tubes (on ice). (Master Mix 1 contains dNTPs, primers, and template DNA; Master Mix 2 contains GC-RICH PCR enzyme mix and reaction buffer.)  
 For each mix, add the components in the order listed below. Numbered vials are supplied with the kit.

##### Master Mix 1 (for one reaction)

Components (Mix 1)	Volume	Final conc.
PCR Grade water (vial 5)	variable	
PCR Grade Nucleotide Mix, 10 mM	1 $\mu$ l	200 $\mu$ M (each nucleotide)
Downstream primer	variable	200 nM
Upstream primer	same as downstream primer	200 nM
Template DNA or cDNA	variable	10 – 500 ng DNA 1 – 100 ng cDNA 0.1 – 1.0 ng plasmid DNA
<b>Final volume (Mix 1)</b>	<b>35 <math>\mu</math>l</b>	

a) To prepare Master Mix 1 for more than one reaction, multiply the amounts of all components of Master Mix 1 (except template DNA) by Z, where Z = the number of reactions to be run + one additional reaction. Mix these components to form a homogeneous solution, aliquot equal amounts of the solution to new 1.5 ml microfuge tubes (one for each reaction, on ice), add template DNA to each, and label each new tube "Master Mix 1 + (template name)".

##### Master Mix 2 (for one reaction)

Components (Mix 2)	Volume	Final conc.
PCR Grade water (vial 5)	4 $\mu$ l	
5 $\times$ GC-RICH reaction buffer with DMSO (vial 2)	10 $\mu$ l	1.5 mM MgCl <sub>2</sub>
GC-RICH enzyme mix (vial 1)	1 $\mu$ l	2 U/50 $\mu$ l reaction
<b>Final volume (Mix 2)</b>	<b>15 <math>\mu</math>l</b>	

b) To prepare Master Mix 2 for more than one reaction, multiply the amounts of all ingredients by Z, where Z = the number of reactions to be run + one additional reaction.



# 4

- 3 Mix the reagents in each tube and centrifuge briefly.
- 4
  - ▶ For each reaction, combine 35  $\mu$ l Mix 1 and 15  $\mu$ l Mix 2 in a thin-walled PCR tube (or well of a PCR microplate) on ice.
  - ▶ Gently mix the solutions in each PCR tube (or well) to produce a homogeneous reaction mixture.
  - ▶ According to the instructions supplied with your instrument, prepare the tubes or microplate for PCR (e.g., overlay reaction mixture in tube with mineral oil or seal the microplate with adhesive foil).
- 5 Place PCR tubes (or microplate) in the PCR instrument and begin PCR immediately.
  - ! *The completed reaction mixtures should not be stored for extended periods of time.*

### PCR

After placing the samples in the PCR instrument, cycle according to the thermal profile below.

! *The thermal profile below was developed for the Applied Biosystems GeneAmp PCR System 2400 thermal block cycler. Other PCR instruments may require a different profile.*

Cycles		Temperature	Time
<b>Initial Denaturation</b>			
1		95°C	3 min <sup>a</sup>
<b>Amplification Program 1</b>			
10	Denaturation	95°C	30 s
	Annealing	45 to 65°C <sup>b</sup>	30 s
	Elongation	72 or 68°C <sup>c</sup>	45 s/kb <sup>d</sup>
<b>Amplification Program 2</b>			
20–25	Denaturation	95°C	30 s
	Annealing	45 to 65°C <sup>b</sup>	30 s
	Elongation	72 or 68°C <sup>c</sup>	45 s/kb <sup>d</sup> + 5 s cycle elongation for each successive cycle <sup>e</sup>
<b>Final Elongation</b>			
1		68°C	7 min

<sup>a)</sup> GC-rich templates require 95°C for 3 min. Templates with mixed GC content may only require a 2 min incubation at 95°C.

<sup>b)</sup> Optimal annealing temperature depends on the melting temperature of the primers and the system used.

<sup>c)</sup> Use 72°C for fragments up to 3 kb. For fragments >3 kb, use 68°C.

<sup>d)</sup> Elongation time depends on fragment length. Use 45 s/kb for fragments up to 5 kb.

<sup>e)</sup> Gradually increasing extension time ensures a higher yield of amplification products. For example, cycle no. 11 is 5 s longer than cycle 10, cycle no. 12 is 10 s longer than cycle 10, cycle no. 13 is 15 s longer than cycle 10, etc.

## Protocol K: Amplification with the Help of the GC-RICH Resolution Solution

### Setting Up the Reaction

- 1 ▶ Thaw all frozen reagents before use.  
 ▶ Vortex all reagents thoroughly and briefly centrifuge them before starting the procedure.
- 2 Prepare two separate master mixes in sterile 1.5 ml microfuge tubes (on ice). (Master Mix 1 contains dNTPs, primers, and template DNA; Master Mix 2 contains GC-RICH PCR enzyme mix and reaction buffer.)  
 For each mix, add the components in the order listed below. Numbered vials are supplied with the kit.

#### Master Mix 1 (for one reaction) <sup>a</sup>

Components (Mix 1)	Volume	Final conc.
PCR Grade water (vial 5)	variable	
PCR Grade Nucleotide Mix, 10 mM	1 µl	200 µM (each nucleotide)
Downstream primer	variable	200 nM
Upstream primer	same as downstream primer	200 nM
Template DNA or cDNA	variable	10–500 ng DNA 1–100 ng cDNA 0.1 – 1.0 ng plasmid DNA
GC-RICH Resolution Solution (vial 3)	5–25 µl	0.5–2.5 M  Determine concentration by titration. See below.
<b>Final volume (Mix 1)</b>	<b>35 µl</b>	

<sup>a</sup> To prepare Master Mix 1 for more than one reaction, multiply the amounts of all components of Master Mix 1 (except template DNA) by Z, where Z = the number of reactions to be run + one additional reaction. Mix these components to form a homogeneous solution, aliquot equal amounts of the solution to new 1.5 ml microfuge tubes (one for each reaction, on ice), add template DNA to each, and label each new tube “Master Mix 1 + (template name)”.

#### Master Mix 2 (for one reaction)

Components (Mix 2)	Volume	Final conc.
PCR Grade water (kit vial 5)	4 µl	
5× GC-RICH reaction buffer with DMSO (vial 2)	10 µl	1.5 mM MgCl <sub>2</sub>
GC-RICH enzyme mix (vial 1)	1 µl	2 U/50 µl reaction
<b>Final volume (Mix 2)</b>	<b>15 µl</b>	

- 3 Mix the reagents in each tube and centrifuge briefly.
- 4 ▶ For each reaction, combine 35 µl Mix 1 and 15 µl Mix 2 in a thin-walled PCR tube (or well of a microplate) on ice.  
 ▶ Gently mix the solutions in each PCR tube (or well) to produce a homogeneous reaction mixture.  
 ▶ According to the instructions supplied with your instrument, prepare the tubes or microplate for PCR (e.g., overlay reaction mixture in tube with mineral oil or seal the microplate with adhesive foil).
- 5 Place PCR tubes (or microplate) in the PCR instrument and begin PCR immediately.  
 The completed reaction mixtures should not be stored for extended periods of time.

4

### Titrating GC-RICH Resolution Solution

For each template, determine the optimal concentration of GC-RICH Resolution Solution by titration. Follow these guidelines for the titration:

Final Conc., Resol. Soln (M)	0.5	1.0	1.5	2.0	2.5
Volume of vial 3 added to Master Mix 1 (for 50 $\mu$ l reaction mix)	5 $\mu$ l	10 $\mu$ l	15 $\mu$ l	20 $\mu$ l	25 $\mu$ l



*GC-RICH Resolution Solution can be added to Master Mix 2 (along with the GC-RICH reaction buffer and enzyme mix) instead of Master Mix 1. In that case, the final volume of Master Mix 2 would be 35  $\mu$ l and the final volume of Master Mix 1 would be 15  $\mu$ l.*

### PCR

Use the same PCR program as in Protocol A above.

## Troubleshooting the Amplification

### Optimizing Reaction Conditions

For initial experiments, use the conditions listed in the protocols above, which generally give good results. However, if initial results are unsatisfactory, use the following guidelines to optimize the critical components of the reaction:

Parameter	Optimal Concentration or Condition
Template DNA	10 – 500 ng human genomic DNA, or 1 – 100 ng cDNA 0.1 – 1.0 ng plasmid DNA
Primers	between 200 and 500 nM (final concentration of each primer)
MgCl <sub>2</sub>	between 1 and 4 mM
GC-RICH Enzyme Mix	between 0.5 and 5.0 U per assay



*Optimal reaction conditions (reagent concentrations, incubation times and temperatures, etc.) vary from system to system. For general information on optimizing a PCR reaction, see section 4.7, “Guidelines for Optimizing PCR.”*

### More Troubleshooting Options

- ▶ If Protocol A (with GC-RICH reaction buffer alone) does not give the expected yield or specificity with the template, try using Protocol B (with both GC-RICH Resolution Solution and reaction buffer).
- ▶ If initial experiments with Protocol B produce nonspecific amplification products, repeat the protocol but increase the concentration of the GC-RICH Resolution Solution in 0.5 M steps, up to a maximum of 2.5 M.



*For additional troubleshooting options, see “Troubleshooting” in the Appendix of this manual.*

## Processing of PCR Products

### Analysis

Analyze the products on a 1–2% agarose gel.

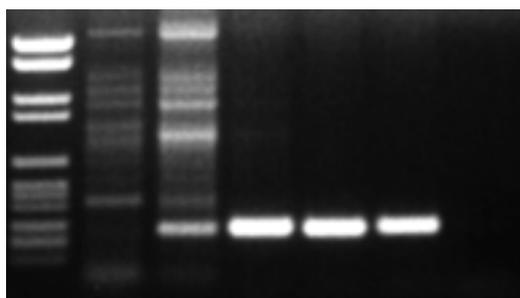
### Cloning

We recommend TA cloning. PCR products generated with the GC-RICH PCR System usually have a single A overhang (3' end), although some may have blunt ends.

## 4.4.4 Typical Results

**Result:** Taq DNA Polymerase alone could not amplify the ApoE template at all (result not shown). The Expand High Fidelity PCR System did not amplify the ApoE template specifically; it produced only nonspecific products (lane 1). The GC-RICH PCR System, on the other hand, produced good yields of the specific 264 bp product when the reaction was supplemented with 0.5–1.5 M GC-RICH Resolution Solution (lanes 3–5).

1      2      3      4      5      6      7



**Figure 4.4.1. Amplification of GC-Rich Templates with the GC-RICH PCR System – Comparison with other PCR Enzymes and Effect of GC-RICH Resolution Solution.** A 264 bp template (GC content, 74%), derived from the human ApoE gene, was amplified through 30 cycles of PCR under varying reaction conditions, as detailed below. Products from each reaction were analyzed on a 1% LE-Agarose gel.

The enzyme, protocol, and reaction supplement used to generate each product were:

**Lane 1:** Molecular Weight Markr VI\*

**Lane 2:** Expand High Fidelity PCR System, 2.6 units; Expand High Fidelity protocol, no supplement

**Lane 3:** GC-RICH PCR System, 2.0 units; Protocol A without GC-RICH Resolution Solution

**Lane 4:** GC-RICH PCR System, 2.0 units; Protocol B with 0.5 M GC-RICH Resolution Solution

**Lane 5:** GC-RICH PCR System, 2.0 units; Protocol B with 1.0 M GC-RICH Resolution Solution

**Lane 6:** GC-RICH PCR System, 2.0 units; Protocol B with 1.5 M GC-RICH Resolution Solution

**Lane 7:** GC-RICH PCR System, 2.0 units; Protocol B with 2.0 M GC-RICH Resolution Solution

# 4

## 4.5 Preventing Carryover

### Purpose of Procedure:

Elimination of carryover contamination (*i.e.*, from previously amplified DNAs) during amplification of normal DNA targets up to 5 kb long.

### Enzyme/System Needed:

PCR Core Kit<sup>PLUS</sup> (for targets up to 3 kb), or

Expand High Fidelity<sup>PLUS</sup> PCR System (for targets up to 5 kb)



*For more information on choosing the correct amplification procedure, see the PCR Protocol Selection Guide at the beginning of Chapter 4.*

### Mechanism for Preventing Carryover Contamination

Including uracil-DNA N-glycosylase (UNG) and deoxyuridine triphosphate (dUTP) in all PCRs can help prevent carryover contamination (*i.e.*, contamination with previously amplified PCR products). The prevention procedure involves incorporating dUTP during PCR so the amplicons contain deoxyuracil (dU), then pretreating subsequent PCR mixtures with UNG. If a dU-containing contaminant from a previous PCR is present in a new PCR, it will be cleaved by a combination of UNG digestion and the high temperature of the initial denaturation step; after treatment, it cannot serve as a PCR template. Since any newly added DNA template (*e.g.*, your target DNA) contains thymidine rather than uridine, it is not affected by this procedure.

### 4.5.1 Reagents and Equipment Required

#### Reagent Kit Required

The protocols in section 4.5.3 below use the following kits to prevent carryover during amplification of genomic or plasmid DNA targets:

- ▶ For amplification of targets up to 3 kb long: Use the PCR Core Kit<sup>PLUS</sup>.
- ▶ For amplification of targets up to 5 kb long: Use the Expand High Fidelity<sup>PLUS</sup> PCR System.



*For pack sizes, see Ordering Information in the Appendix.*

#### Additional Equipment and Reagents Required

- ▶ Thermal block cycler (*e.g.*, Applied Biosystems GeneAmp PCR System 2400) or other PCR instrument
- ▶ 0.2 ml thin-walled PCR tubes\* or PCR microplate, suitable for your PCR instrument
- ▶ Sterile reaction tubes for preparing master mixes and dilutions
- ▶ PCR Grade Water \*
- ▶ PCR Grade Nucleotide Mix<sup>PLUS</sup> \* (for protocol M only)

\* available from Roche Applied Science; see Ordering Information (in the Appendix) for details.

## 4.5.2 General Considerations for Preventing Carryover

### Using Other PCR Enzymes in Carryover Prevention Protocols

Deoxyuridine triphosphate (dUTP) is incorporated readily and efficiently by some PCR enzymes, but not by others. In particular, dUTP binds very tightly to Type B polymerases (e.g., Pwo DNA Polymerase) and strongly inhibits amplification by these enzymes. The table below shows which enzymes and enzyme mixes offered by Roche Applied Science are inhibited by dUTP and which are not.

dUTP Strongly Inhibits these Enzymes	dUTP Does Not Inhibit these Enzymes
Pwo DNA Polymerase	Taq DNA Polymerase
Expand High Fidelity enzyme mix <sup>a</sup>	FastStart Taq DNA Polymerase
Expand Long Range enzyme mix <sup>a</sup>	Expand High Fidelity <sup>PLUS</sup> enzyme mix <sup>b</sup>
Expand 20 kb <sup>PLUS</sup> enzyme mix <sup>a</sup>	FastStart High Fidelity PCR System <sup>b</sup>
GC-RICH enzyme mix <sup>a</sup>	

<sup>a)</sup> contains Taq DNA Polymerase and a proofreading, Type B DNA polymerase

<sup>b)</sup> contains Taq DNA Polymerase and a thermostable proofreading protein that lacks polymerase activity

Obviously, those enzymes that are inhibited by dUTP should not be used in carryover prevention protocols such as those described in this section.

### Other Special Notes about Carryover Prevention Protocols

In addition to using a suitable enzyme (see the table above), you should remember the following when you are designing a carryover prevention protocol:

- ▶ The optimal concentration for dUTP in a PCR is generally 3× that of the normal nucleotides (dATP, dCTP, dGTP). For example, if the reaction uses 200 μM for the three normal nucleotides, the optimal concentration of dUTP will be 600 μM.
- ▶ Reactions that contain dUTP also require higher concentrations of Mg<sup>2+</sup>. The suggested MgCl<sub>2</sub> concentration for reactions that contain 600 μM dUTP is 2.5 mM.
- ▶ Not all preparations of UNG are inactivated by the conditions described in these protocols (2 min at 94°C or 95°C). To ensure complete inactivation of the UNG, use the heat-labile preparation of UNG specified in these protocols.
- ▶ Only DNA targets that contain dA and dT will lead to amplicons that contain dU and can be degraded by UNG.
- ▶ Do not use PCR primers that contain dU, since these will be degraded by UNG and not be available to direct the amplification.
  - ⚠ *UNG will not digest 3'-terminal dU, nor will it digest primers labeled with biotin-dU or digoxigenin-dU.*
- ▶ Although DNAs that contain dU work well in most downstream procedures, there are some limitations. For details, see section "Processing of PCR Products" below.



### Sample Material

Any suitable genomic or plasmid DNA. DNA samples should have an average GC content, no unusual sequences, and be as pure as possible.



*In any PCR, the quality of the template greatly influences amplification yield and efficiency. For more information on preparing high quality DNA templates suitable for PCR, see Chapter 3.*

### Template Dilution Buffer

The optimal dilution buffer for template DNA is either sterile, double-distilled water or Tris (5–10 mM, pH 7–8).



*Do not dissolve the template in TE buffer because EDTA chelates Mg<sup>2+</sup>.*

# 4

## 4.5.3 Protocols for Preventing Carryover

### Protocol L: Amplification of up to 3 kb targets with the PCR Core Kit<sup>PLUS</sup>

#### About the PCR Core Kit<sup>PLUS</sup>

The PCR Core Kit<sup>PLUS</sup> contains all the reagents (except water, primers and template) required for carryover prevention protocols. It includes Taq DNA Polymerase\*; heat-labile UNG\*; a special PCR Grade Nucleotide Mix<sup>PLUS</sup>\* (10 mM each of dATP, dCTP and dGTP; 30 mM dUTP); two reaction buffers (one with MgCl<sub>2</sub>, the other without) and a 25 mM MgCl<sub>2</sub> stock solution (for optimizing Mg<sup>2+</sup>).

\* also available as separate reagents from Roche Applied Science; see Ordering Information (in the Appendix) for details.

### Setting Up the Reaction

- 1 ▶ Thaw all frozen reagents before use.  
 ▶ Mix all reagents and briefly centrifuge them before starting the procedure.
- 2 Prepare two separate master mixes in sterile 1.5 ml microfuge tubes (on ice). (Master Mix 1 contains dNTPs, primers, and template DNA; Master Mix 2 contains PCR buffer and enzymes.)  
 For each mix, add the components in the order listed below. Numbered vials are supplied in the kit.

#### Master Mix 1 (for one reaction) <sup>a</sup>

Components (Mix 1)	Volume	Final conc.
PCR Grade water	variable	
PCR Grade Nucleotide Mix <sup>PLUS</sup> (vial 3)	1 µl	200 µM dATP, dCTP, dGTP 600 µM dUTP
Downstream primer	variable	200 nM
Upstream primer	same as downstream primer	200 nM
Template DNA	variable	100–500 ng complex DNA 1–10 ng plasmid DNA
<b>Final volume (Mix 1)</b>	<b>25 µl</b>	

<sup>a</sup>) To prepare Master Mix 1 for more than one reaction, multiply the amounts of all components of Master Mix 1 (except template DNA) by Z, where Z = the number of reactions to be run + one additional reaction. Mix these components to form a homogeneous solution, aliquot equal amounts of the solution to new 1.5 ml microfuge tubes (one for each reaction, on ice), add template DNA to each, and label each new tube “Master Mix 1 + (template name)”.

#### Master Mix 2 (for one reaction) <sup>b</sup>

Components (Mix 2)	Volume	Final conc.
PCR Grade water	16.75 µl	
Core Kit Buffer + MgCl <sub>2</sub> , 10× conc. (vial 4)	5 µl	2.5 mM MgCl <sub>2</sub>
Taq DNA Polymerase, 5 units/µl (vial 1)	0.25 µl	2.5 U per 50 µl reaction
UNG, heat-labile, 1 unit/µl	2 µl	2 U per 50 µl reaction
<b>Final volume (Mix 2)</b>	<b>25 µl</b>	

<sup>b</sup>) To prepare Master Mix 2 for more than one reaction, multiply the amounts of all ingredients by Z, where Z = the number of reactions to be run + one additional reaction.

- 3 Mix the reagents in each tube and centrifuge briefly.
- 4 ▶ For each reaction, combine 25 µl Mix 1 (including DNA template) and 25 µl Mix 2 in a thin-walled PCR tube (or well of a PCR microplate) on ice.  
 ▶ Gently mix the solutions in each PCR tube (or well) to produce a homogeneous reaction mixture.  
 ▶ According to the instructions supplied with your instrument, prepare the tubes or microplate for PCR (e.g., overlay reaction mixture in tube with mineral oil or seal the microplate with adhesive foil).
- 5 Place PCR tubes (or microplate) in the PCR instrument and begin PCR immediately.  
 The completed reaction mixtures should not be stored for extended periods of time.



## PCR

After placing the samples in the PCR instrument, cycle according to the thermal profile below.



The thermal profile below was developed for the Applied Biosystems GeneAmp PCR System 2400 thermal block cycler. Other PCR instruments may require a different profile.

Cycles		Temperature	Time
<b>Pre-PCR Steps <sup>a</sup></b>			
1	UNG incubation	20°C	1–10 min <sup>b</sup>
	Inactivation of UNG	95°C	2 min
<b>Amplification Program 1</b>			
10	Denaturation	95°C	10 s
	Annealing	55 to 65°C <sup>c</sup>	30 s
	Elongation	72°C	1.5 min <sup>d</sup>
<b>Amplification Program 2</b>			
20	Denaturation	95°C	10 s
	Annealing	55 to 65°C <sup>c</sup>	30 s
	Elongation	72°C <sup>e</sup>	1.5 min <sup>d</sup> + 20 s cycle elongation for each successive cycle <sup>e</sup>
<b>Final Elongation</b>			
1		72°C	7 min

- <sup>a)</sup> Primer-dimers may form during the UNG incubation at 20°C. If primer-dimers form in initial experiments, modify the protocol to allow a hot start (see the Troubleshooting section below).
- <sup>b)</sup> Two units of UNG will degrade up to 10<sup>6</sup> dU-containing templates in 10 min.
- <sup>c)</sup> Optimal annealing temperature depends on the melting temperature of the primers and the system used.
- <sup>d)</sup> Elongation time depends on fragment length. A 1.5 min elongation is sufficient for a 3 kb fragment.
- <sup>e)</sup> Gradually increasing extension time ensures a higher yield of amplification products. For example, cycle no. 11 is 20 s longer than cycle 10, cycle no. 12 is 40 s longer than cycle 10, cycle no. 13 is 60 s longer than cycle 10, etc.

4

## Protocol M: Amplification of up to 5 kb targets with the Expand High Fidelity<sup>PLUS</sup> System

### About the Expand High Fidelity<sup>PLUS</sup> System

The Expand High Fidelity<sup>PLUS</sup> System contains a novel mixture of Taq DNA Polymerase and a thermostable proofreading protein that lacks polymerase activity. Unlike other PCR enzymes or enzyme mixtures with proofreading activity, the Expand High Fidelity<sup>PLUS</sup> mixture readily incorporates dUTP into amplicons. Thus, the Expand High Fidelity<sup>PLUS</sup> System will amplify DNA targets up to 5 kb long with outstanding yield and fidelity, and also work with UNG to prevent carryover contamination.

 *The proteins in the Expand High Fidelity<sup>PLUS</sup> System are not the same as the ones in the Expand High Fidelity System. The Expand High Fidelity System contains a Type B DNA polymerase, which is inhibited by dUTP. Thus, the Expand High Fidelity System should not be used in carryover prevention protocols such as those described in this section.*

Also included in the Expand High Fidelity<sup>PLUS</sup> System are other reagents that simplify carryover prevention protocols. These include two reaction buffers (one with MgCl<sub>2</sub>, the other without) and a 25 mM MgCl<sub>2</sub> stock solution (for optimizing Mg<sup>2+</sup>).

### Setting Up the Reaction

- 1  Thaw all frozen reaction components before use.  
 Mix all reagents and briefly centrifuge them before starting the procedure.
- 2 Prepare two separate master mixes in sterile 1.5 ml microfuge tubes (on ice). (Master Mix 1 contains dNTPs, primers, and template DNA; Master Mix 2 contains PCR buffer and enzymes.)  
 *For each mix, add the components in the order listed below. Numbered vials are supplied in the kit.*

#### Master Mix 1 (for one reaction)

Components (Mix 1)	Volume <sup>a</sup>	Final conc.
PCR Grade water	variable	
PCR Grade Nucleotide Mix <sup>PLUS</sup>	1 µl	200 µM dATP, dCTP, dGTP 600 µM dUTP
Downstream primer	variable	200 nM
Upstream primer	same as downstream primer	200 nM
Template DNA	variable	5–500 ng complex DNA 0.1–10 ng plasmid DNA
<b>Final volume (Mix 1)</b>	<b>25 µl</b>	

<sup>a)</sup> To prepare Master Mix 1 for more than one reaction, multiply the amounts of all components of Master Mix 1 (except template DNA) by Z, where Z = the number of reactions to be run + one additional reaction. Mix these components to form a homogeneous solution, aliquot equal amounts of the solution to new 1.5 ml microfuge tubes (one for each reaction, on ice), add template DNA to each, and label each new tube “Master Mix 1 + (template name)”.



**Master Mix 2** (for one reaction)

Components (Mix 2)	Volume <sup>b</sup>	Final conc.
PCR Grade water	12.5 µl	
Expand HiFi <sup>PLUS</sup> Buffer + MgCl <sub>2</sub> , 5× conc. (vial 4)	10 µl	1.5 mM MgCl <sub>2</sub>
Expand HiFi <sup>PLUS</sup> Enzyme Mix, 5 U/µl (vial 1)	0.5 µl	2.5 U per 50 µl reaction
UNG, heat-labile, 1 unit/µl	2 µl	2 units per 50 µl reaction
<b>Final volume (Mix 2)</b>	<b>25 µl</b>	

<sup>b)</sup> To prepare Master Mix 2 for more than one reaction, multiply the amounts of all ingredients by Z, where Z = the number of reactions to be run + one additional reaction.

- 3** Mix the reagents in each tube and centrifuge briefly.
- 4**
  - ▶ For each reaction, combine 25 µl Mix 1 (including DNA template) and 25 µl Mix 2 in a thin-walled PCR tube (or well of a PCR microplate) on ice.
  - ▶ Gently mix the solutions in each PCR tube (or well) to produce a homogeneous reaction mixture.
  - ▶ According to the instructions supplied with your instrument, prepare the tubes or microplate for PCR (e.g. overlay reaction mixture in tube with mineral oil or seal the microplate with adhesive foil.)
- 5** Place PCR tubes (or microplate) in the PCR instrument and begin PCR immediately.  
 *The completed reaction mixtures should not be stored for extended periods of time.*

# 4

## PCR

Place samples in the PCR instrument, then cycle according to the thermal profile below.

 The thermal profile below was developed for the Applied Biosystems GeneAmp PCR System 2400 thermal block cycler. Other PCR instruments may require a different profile.

Cycles		Temperature	Time
<b>Pre-PCR Steps</b>			
1	UNG incubation	20°C	10 min <sup>a</sup>
	Inactivation of UNG	94°C	2 min
<b>Amplification Program 1</b>			
10	Denaturation	94°C	10–30 s <sup>b</sup>
	Annealing	55 to 68°C <sup>c</sup>	30 s
	Elongation	72 or 68°C <sup>d</sup>	1–5 min <sup>e</sup>
<b>Amplification Program 2</b>			
20	Denaturation	94°C	10–30 s <sup>b</sup>
	Annealing	55 to 68°C <sup>c</sup>	30 s
	Elongation	72 or 68°C <sup>d</sup>	1–5 min <sup>e</sup> + 10 s cycle elongation for each successive cycle <sup>f</sup>
<b>Final Elongation</b>			
1		72°C	7 min

- <sup>a)</sup> Two units of UNG will degrade up to 10<sup>6</sup> dU-containing templates in 10 min. It may be possible to shorten this incubation time, thus minimizing the formation of primer-dimers.
- <sup>b)</sup> Optimal denaturation time depends on the GC content of the template, with higher amounts of GC requiring longer incubations. Use the shortest denaturation time that will completely denature your template (as determined in separate experiments).
- <sup>c)</sup> Optimal annealing temperature depends on the melting temperature of the primers and the system used.
- <sup>d)</sup> For PCR products up to 3 kb long, use 72°C for elongation. For PCR products longer than 3 kb, use 68°C.
- <sup>e)</sup> Elongation time depends on product length. Use 1 min per kb for products up to 5 kb long.
- <sup>f)</sup> Gradually increasing extension time ensures a higher yield of amplification products. For example, cycle no. 11 is 10 s longer than cycle 10, cycle no. 12 is 20 s longer than cycle 10, cycle no. 13 is 30 s longer than cycle 10, etc.

### Troubleshooting the Amplification

- If initial experiments produce unsatisfactory yields, try increasing the Mg<sup>2+</sup> concentration in 0.5 mM steps. You may use Mg<sup>2+</sup> concentrations up to 4 mM. To increase the Mg<sup>2+</sup> concentration in either protocol, use the reaction buffer without MgCl<sub>2</sub> supplied in the kit (vial 5 in the PCR Core Kit<sup>PLUS</sup>, vial 3 in the Expand High Fidelity<sup>PLUS</sup> System) and the 25 mM MgCl<sub>2</sub> solution (vial 6 in the PCR Core Kit<sup>PLUS</sup>, vial 4 in the Expand High Fidelity<sup>PLUS</sup> System) to produce the following concentrations:

**If you add the following volume of 25 mM MgCl<sub>2</sub> stock to a 50 µl PCR mixture:**

4 µl    4.5 µl    5 µl    5.5 µl    6 µl    6.5 µl    7 µl    7.5 µl    8 µl

**You will have a final MgCl<sub>2</sub> reaction concentration of:**

2 mM    2.25 mM    2.5 mM    2.75 mM    3 mM    3.25 mM    3.5 mM    3.75 mM    4 mM

4

# 4

- ▶ If initial experiments result in extra, unexpected PCR products, these may be primer-dimers. To eliminate primer-dimers in the procedure, either decrease the time allowed for the UNG incubation, or use a hot start technique by modifying Protocol A (above) as follows:
- ▶ Substitute 2 units of FastStart Taq DNA Polymerase (available from Roche Applied Science) for the regular Taq DNA Polymerase in Master Mix 2.
- ▶ Reduce the UNG in Master Mix 2 to 1 unit (1  $\mu$ l).
- ▶ In the Pre-PCR Steps part of the PCR program, allow 4 min for inactivation of UNG. The increased incubation time at 95°C allows the FastStart Taq DNA Polymerase to be fully activated.
- ▶ In Amplification Programs 1 and 2, increase the denaturation step to 30 s and allow about 1 min/kb for each amplification step (e.g., 3 min for a 3 kb target).
- ▶ In Amplification Program 2, increase the elongation time by 5 s per cycle. For example, cycle no. 11 is 5 s longer than cycle 10, cycle no. 12 is 10 s longer than cycle 10, cycle no. 13 is 15 s longer than cycle 10, etc.



For additional troubleshooting options, see “Troubleshooting” in the Appendix of this manual.

## Processing of PCR Products

### Analysis

Analyze the products on a 1–2% agarose gel.

### Storage

Heat-labile UNG will not degrade a dU-containing DNA for several hours at 4°C. PCR products may be stored for up to 8 h at 4°C (e.g., in the PCR instrument). For periods longer than 8 h, store the products frozen at -15 to -25 °C.

### Digestion with Restriction Enzymes

Some common restriction enzymes (e.g., *Eco*RI and *Bam*HI) readily digest DNA that contains dU. However, other restriction enzymes (e.g., *Hpa*I and *Hind*II, *Hind*III) digest dU-containing DNA more slowly than normal substrates. Before digesting large amounts of your substrate with a given restriction enzyme, perform a small-scale digestion to determine whether to increase the amount of enzyme in the reaction.

### Cloning

We recommend TA cloning. PCR products generated with Taq DNA Polymerase usually have a single A overhang (3' end).

### Putting Amplicon into Bacteria

Always use *ung*<sup>r</sup> bacterial hosts when transforming PCR products that contain dU.

### Other Techniques

Do not use DNA that contains dU for protein binding or studies of DNA-protein interactions.

## 4.6 Guidelines for Optimizing PCR

In principle, the protocols listed in sections 4.1 – 4.6 will give excellent results with a wide variety of templates and targets. However, when the results obtained in initial experiments are not satisfactory, you may need to optimize the PCR. Below are some general guidelines for optimization.

### 4.6.1 Choose the Appropriate Enzyme

Roche Applied Science supplies a wide variety of PCR enzymes and enzyme systems. Although all give excellent results, each enzyme (or enzyme system) is optimized for templates with different characteristics (e.g., high GC content or limited availability) and products of different lengths. In addition, some enzyme systems are more suitable for certain applications (e.g., for mutation studies or multiplexing). Therefore, if one enzyme preparation yields less than satisfactory results, try another that may be better suited to your experimental system.



*For guidelines on selecting the right enzyme for your application, see the PCR Protocol Selection Guide at the beginning of Chapter 4.*

### 4.6.2 Use Highly Purified Templates and Primers

In principle, the template may be any kind of DNA (plasmid, genomic DNA, cDNA) that is suitable for PCR, as long as it is sufficiently pure, concentrated and free of PCR inhibitors.



*Be cautious when using cDNA as template. Do not let the volume of the cDNA template exceed 10% of the volume of the PCR mixture. For more information on using cDNA as PCR template, see section 5.4, “Two-Step RT-PCR,” in this manual.*



*The quality of the template greatly influences the outcome of the PCR. For information on preparing high quality templates that give optimal PCR results, see Chapter 3.*

### 4.6.3 Design Primers Carefully

The design of the PCR primers determines amplicon length, melting temperature, amplification efficiency, and yield. When designing suitable primers for your application, remember these guidelines:

- ▶ Primer design may be influenced by the choice of the PCR program (2-step vs. 3-step protocol).
- ▶ Avoid primer sequences that could form secondary structures or dimers.
- ▶ In the reaction mix, you should use equal concentrations of the two primers.
- ▶ The primers should be of similar length. For example, to amplify long targets (20–35 kb), the primers should each be 22–34 nucleotides long.

- ▶ To ensure a specific and sensitive assay, the primer must anneal to and melt off the DNA at suitable temperatures. For example:
  - ▶ To amplify long targets (20–35 kb), the primers should have melting temperatures >60°C.
  - ▶ If they are to be used in a quantitative assay with detection probes, the primers should anneal to the target at a temperature significantly higher than the annealing temperature of the probe.
- ▶ The primers should have similar annealing temperatures.



*Several programs for primer design are provided by the suppliers of real-time PCR instruments (e.g., PrimerExpress). Alternatively, such programs are available for free on the web [e.g., Primer3 or ProbeFinder™ (which also uses the Primer3 program)].*

The advantage of the ProbeFinder™ software ([www.universalprobelibrary.com](http://www.universalprobelibrary.com)) is that primers designed with this program may easily be paired with real-time PCR probes from the Universal ProbeLibrary (available from Roche Applied Science).

#### 4.6.4 Use the Highest Quality Nucleotides

Nucleotides are vital components in amplification reactions and the purity of these reagents significantly influences PCR results.

If conventional procedures are used to synthesize nucleotides, different contaminants (pyrophosphate, mono-, di- and tetraphosphate nucleotides and organic solvents) remain in the preparation after the synthesis reaction. Since trace amounts of these contaminants inhibit amplification reactions, PCR nucleotides should be absolutely free of them. Thus, conventionally generated nucleotides are suboptimal for PCR.

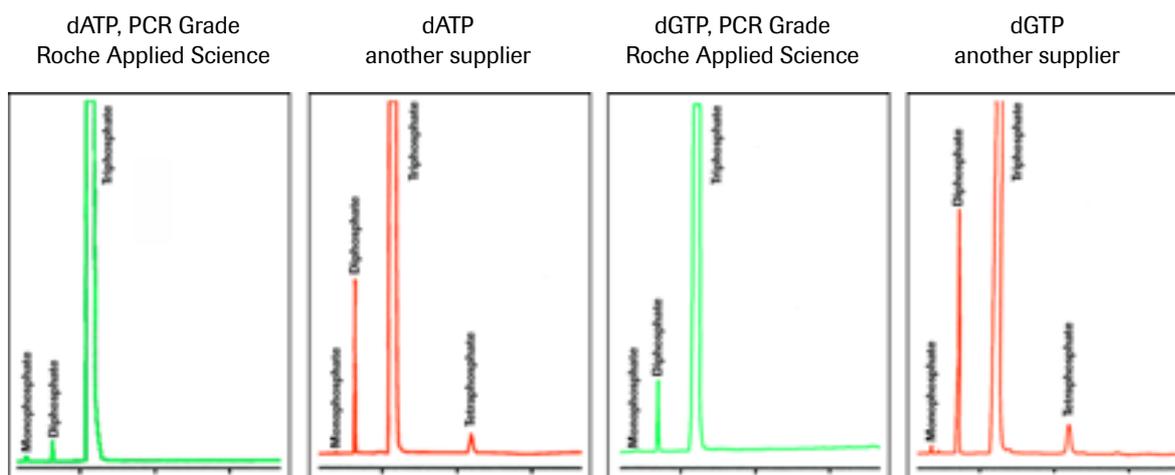
To solve this problem, Roche Applied Science developed a new process to synthesize nucleotides. This process results in a drastically reduction of the amount of contaminants, streamlines the purification process and delivers nucleotides that are virtually free of all contaminants (Figure 4.6.1).

These high quality (“PCR Grade”) nucleotides are now accepted in the scientific community as the standard nucleotides that should be used for PCR.

Since nucleotides only contribute approx. 1 - 2% to the total cost of a PCR reaction, buying nucleotides from low cost suppliers will not save very much money. However, considerable costs can be incurred if PCRs are inhibited by impure nucleotides and have to be repeated.



*PCR Grade Nucleotides are available separately and as part of convenient enzyme-dNTPacks. For a complete listing of our PCR Grade Nucleotide Product Family, see the Ordering Information (in the Appendix).*



**Figure 4.6.1. Purity of PCR Grade Nucleotides.** PCR Grade Nucleotides from Roche Applied Science and nucleotides from another supplier were chromatographically analyzed under conditions that separate mono-, di-, tri- and tetraphosphates.

**Conclusion:** PCR Grade Nucleotides from Roche Applied Science are very pure (>99% of dNTP molecules, with little or no contamination). In contrast, nucleotides from another major supplier are contaminated with significant amounts of di- and tetraphosphates.

#### 4.6.5 Minimize Pipetting Steps with Convenient Master Reagent Mixes

Minimizing the pipetting steps required for preparing PCR mixes not only saves time, but also reduces the chance of introducing contaminants. The easiest way to minimize pipetting steps is to use reagent mixes available from Roche Applied Science, such as the PCR Core Kit or one of the convenient “Masters” (e.g., PCR Master, FastStart PCR Master, High Fidelity PCR Master). These products:

- ▶ Are optimized for a particular group of PCR applications (as indicated by the name of the product) and can be used without time-consuming optimization of individual components
- ▶ Are ready-to-use, concentrated master reagent mixes that contain all the reagents (except nucleic acids such as primer and template) needed for PCR
- ▶ Add several vital components (buffer,  $Mg^{2+}$ , nucleotides, PCR enzyme) to the reaction in a single pipetting step
- ▶ Contain only the purest components (such as PCR Grade nucleotides)
- ▶ May be available in several formulations (e.g., with and without a reference dye such as ROX) for added flexibility
- ▶ May contain dUTP, which can help prevent carryover contamination (when used with uracil-DNA N-glycosylase, UNG, as described in “Preventing Carryover” in Chapter 2, this manual)



For a complete listing of the PCR Core Kit and the various Master Reagent Mixes available, see the Ordering Information (in the Appendix). For more information on the performance of these mixes, see Ziebolz (2003).

4

## 4.6.6 Optimize the Reaction Components

The reaction conditions listed in the protocols give good results with most templates. However, optimal reaction conditions vary from system to system. For best results, you should always determine the optimum parameters for each system.

 *If you are using Taq DNA Polymerase, the easiest way to optimize the most critical reaction parameters is to use the PCR Optimization Kit (available from Roche Applied Science), as outlined below. For recommendations on optimizing reactions involving other PCR enzymes, see the individual protocols that use those enzymes.*

### Protocol N: Optimizing the Mg<sup>2+</sup> Concentration for the Standard PCR Protocol

If you optimize no other reaction component, at least optimize the Mg<sup>2+</sup> concentration. This component can have an enormous effect on the efficiency, yield and specificity of the reaction. To optimize the Mg<sup>2+</sup> concentration (and the reaction pH at the same time) for standard PCRs, perform the following experiment with PCR Optimization Buffers from the PCR Optimization Kit.

 *Vials 1–16 of the PCR Optimization Kit are PCR Optimization Buffers, which contain 100 mM Tris-HCl (adjusted to different pHs), 500 mM KCl and varying amounts of MgCl<sub>2</sub>. When diluted tenfold (in the final reaction mix), the buffers have the following pHs and Mg<sup>2+</sup> concentrations:*

pH	MgCl <sub>2</sub> Concentration (mM)			
	1.0	1.5	2.0	2.5
8.3	A	B	C	D
8.6	E	F	G	H
8.9	I	J	K	L
9.2	M	N	O	P

### Additional Reagents and Equipment Needed

- ▶ Taq DNA Polymerase, 1 U/μl or 5 U/μl, sufficiently concentrated to be used at optimal concentration (typically 1.25 U per 50 μl reaction, but can range from 0.5 to 5 U per reaction)
- ▶ PCR Grade Nucleotide Mix\*, which contains 10 mM of each dNTP
- ▶ PCR Grade Water\*
- ▶ Template DNA, optimal amount (typically 100–500 ng genomic DNA or 10–100 ng cDNA or 10–100 ng plasmid DNA per 50 μl reaction)
- ▶ Primers, optimal concentration (typically 200 nM in the final reaction, but can range from 100 to 600 nM)
- ▶ PCR instrument ( e.g., Applied Biosystems GeneAmp PCR System 2400 thermal block cyclor)

- ▶ 0.2 ml thin-walled PCR tubes\*
- ▶ Sterile 1.5 ml reaction tubes for preparing master mixes and dilutions

\* available from Roche Applied Science; see Ordering Information (in the Appendix) for details.

### Mg<sup>2+</sup> Optimization Procedure

- 1 ▶ Thaw all reaction components before use.  
▶ Mix all reagents thoroughly and briefly centrifuge them before starting the procedure.

- 2 Prepare a Master Mix in a sterile 1.5 ml microfuge tube (on ice).

! For the mix, add the components in the order listed below.

#### Master Mix 1 (for one reaction)

Components (Mix 1)	Volume	Final conc.
PCR Grade water	Variable	
dNTP Mix, 10 mM	17 µl	200 µM (each nucleotide)
Downstream primer	Variable	200 nM
Upstream primer	same as downstream primer	200 nM
DNA or cDNA	Variable	100 – 500 ng gen. DNA 10 – 100 ng cDNA or plasmid
Taq DNA Polymerase, 1 U/µl	21.25 µl	1.25 U
<b>Final volume (Mix 1)</b>	<b>765 µl</b>	

- 3 ▶ Mix the reagents thoroughly (by pipetting up and down) and centrifuge briefly.  
▶ Add 45 µl Master Mix to each of 16 sterile PCR tubes (on ice).
- 4 ▶ Add 5 µl of one of the PCR Optimization Buffers (A – P) to each of the tubes that contains Master Mix.  
▶ Gently mix the reagents in each tube.
- 5 ▶ According to the instructions provided with your PCR instrument, prepare the tubes for PCR (e.g., add mineral oil to the tube if the instrument does not have a top heater).  
▶ Place PCR tubes in the PCR instrument and begin PCR immediately.  
! The completed reaction mixtures should not be stored for extended periods of time.  
! The thermal profile below was developed for the Applied Biosystems GeneAmp PCR System 2400. Other PCR instruments may require a different profile.
- 6 Analyze the products from each tube on a 1 – 2% agarose gel.
- 7 ▶ Determine which of the PCR Optimization Buffers generated the maximum specificity and product yield.  
▶ Use the MgCl<sub>2</sub> concentration and pH from that buffer for all subsequent PCRs performed according to the standard PCR protocol.

4

## PCR

Cycles		Temperature	Time
<b>Initial Denaturation</b>			
1		94°C	2 min
<b>Amplification Program 1</b>			
30	Denaturation	94°C	15 – 30 s
	Annealing	50–65°C <sup>a</sup>	30 – 60 s
	Elongation	72°C	45 s – 2 min <sup>b</sup>
<b>Final Elongation</b>			
1		72°C	7 min

<sup>a)</sup> Optimal annealing temperature depends on the melting temperature of the primers and the system used.

<sup>b)</sup> Elongation time depends on fragment length. Use 45 s for targets that are 1 kb or shorter, 1 min for 1.0 – 1.5 kb targets, 2 min for 1.5 – 3 kb targets.

## Protocol O: Supplement the Standard PCR with Various Additives

You can also test the effect of various additives on standard PCRs with the PCR Optimization Kit, as follows:

### Effect of Additives from the PCR Optimization Kit

- ▶ Thaw all reagents before use.  
▶ Mix all reagents thoroughly and briefly centrifuge them before starting the procedure.
- In a sterile 1.5 ml microfuge tube (on ice), prepare the following Master Mix for testing each additive:  
 For each mix, add the components in the order listed below.

#### Master Mix 1 (for one reaction)

Components (Mix 1)	Volume	Final conc.
Optimal PCR buffer (determined in Step 7 of Procedure A above)	5 µl	1×
dNTP Mix, 10 mM	1 µl	200 µM (each nucleotide)
Downstream primer	Variable	200 nM
Upstream primer	same as downstream primer	200 nM
DNA or cDNA	Variable	same as procedure A above
Taq DNA Polymerase, 1 U/µl	1.25 µl	1.25 U per 50 µl reaction
One or more of the following additives from PCR Optimization Kit:		
100% DMSO (kit vial 17)	0.5 – 5 µl	1 – 10%
Gelatin, 1% (w/v) (kit vial 18)	0.5 – 5 µl	0.01 – 0.1%
Ammonium sulfate, 500 mM (kit vial 19)	0.5 – 3 µl	5 – 30 mM
Glycerol, 50% (v/v) (kit vial 20)	5 – 15 µl	5 – 15%
PCR Grade Water	Variable	to make final volume = 50 µl
<b>Final volume</b>	<b>50 µl</b>	



# 4

- 
- 3 Mix the reagents gently and centrifuge briefly.

---

  - 4
    - ▶ According to the instructions provided with your PCR instrument, prepare the tubes for PCR (e.g., add mineral oil to the tube if the instrument does not have a top heater).
    - ! *The completed reaction mixtures should not be stored for extended periods of time.*
    - ▶ Place PCR tubes in the PCR instrument and begin PCR immediately. Use the thermal profile in procedure A (section G.6. 1) above.

---

  - 5 Analyze the products from each tube on a 1 – 2% agarose gel.

---

  - 6
    - ▶ Determine which additive (or combination of additives) enhance the specificity and yield of the PCR.
    - ▶ Use this additive in all subsequent PCRs performed according to the standard PCR protocol.
- 
- 

#### 4.6.7 Optimize Reaction Temperatures and Times

- ▶ Keep denaturation times as short as possible and denaturation temperature as low as possible.
- ▶ Use more cycles for low abundance targets.
- ▶ Some PCRs, e.g., amplification of long templates, are sensitive to even minute differences in ramping or heat transfer rates of different thermal block cyclers. Therefore, always develop and run your experiment on the same thermal block cycler. If you switch to a different thermal block cycler, adjust the reaction conditions and thermal profile.

#### Disclaimer

For Pwo DNA Polymerase, Cat. Nos. 11 644 947 001, 22 644 955 001; Pwo SuperYield DNA Polymerase, Cat. Nos. 04 340 868 001, 04 340 850 001, and Pwo SuperYield DNA Polymerase, dNTPack, Cat. Nos. 04 743 750 001, 04 743 776 001, and Pwo Master, Cat. No. 03 789 403 001, see Disclaimer No. 2,

For the PCR Master, Cat. No. 11 636 103 001 see Disclaimer No. 3.

For the PCR Core Kit <sup>PLUS</sup>, Cat. No. 11 578 553 001 see Disclaimer No. 4

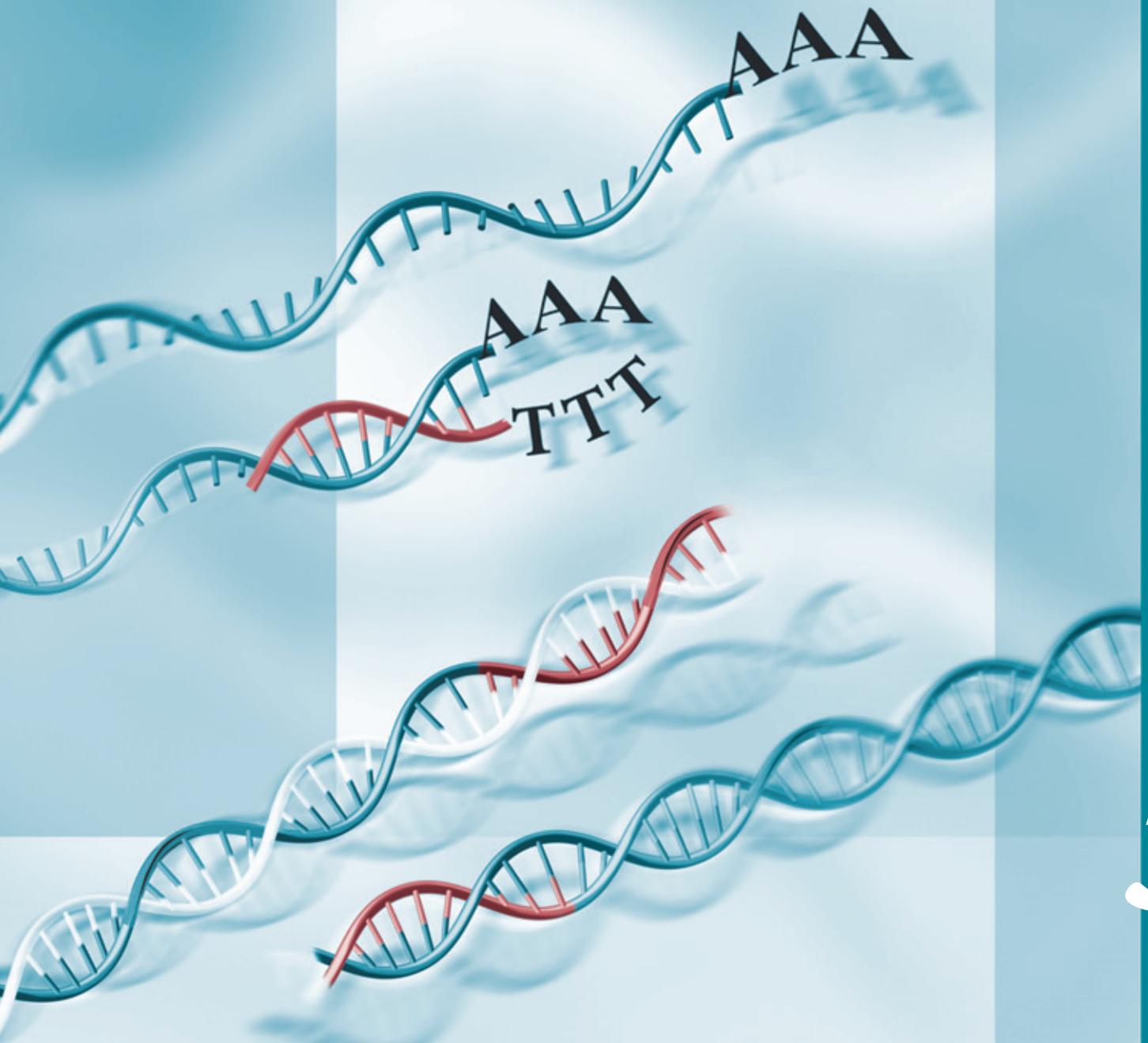


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# 4

*Chapter 5*

# Basic RT-PCR Methods



5

5	Basic RT-PCR Methods	Page
5.1	Factors to Consider in RT-PCR.....	127
5.1.1	Choosing a One-Step or a Two-Step Procedure.....	128
5.1.2	Choosing the RT-PCR Enzymes.....	128
5.1.3	Choosing the Primers for Reverse Transcription.....	130
5.1.4	Preparation and Handling of Template RNA.....	133
5.2	Using Protector RNase Inhibitor.....	134
5.2.1	How To Use Protector RNase Inhibitor.....	134
5.3	One-Step RT-PCR.....	135
5.3.1	Reagents and Equipment Required.....	135
5.3.2	General Considerations for One-Step RT-PCR.....	136
5.3.3	Protocols for One-Step RT-PCR.....	137
5.4	Two-Step RT-PCR.....	145
5.4.1	Reagents and Equipment Required.....	145
5.4.2	General Considerations for Two-Step RT-PCR.....	146
5.4.3	Protocols for the Reverse Transcription Step (Templates up to 14 kb).....	147
5.4.4	Protocols for the PCR Step.....	154
5.4.5	Typical Results of Two-Step RT-PCR.....	155



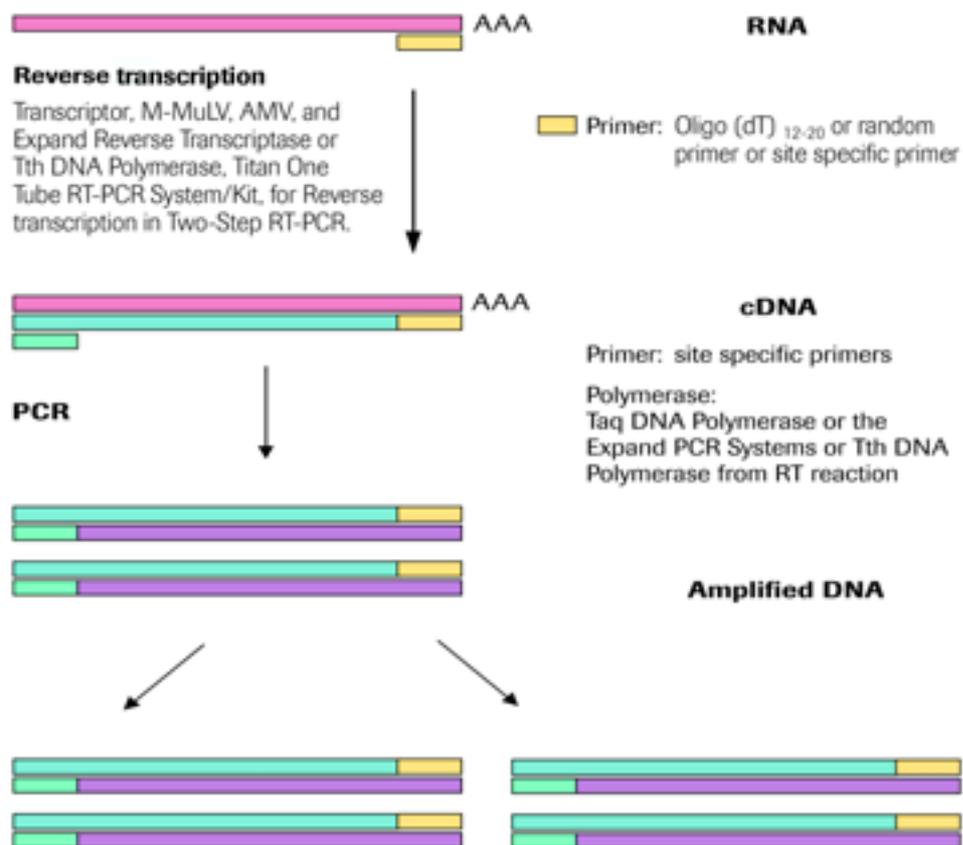
## 5. General RT-PCR Methods

### 5.1 Factors to Consider in RT-PCR

The ability to detect and analyze mRNA transcripts or other RNAs present in low abundance is critical to many experimental studies. In 1987, Powell *et al.* described a technique, derived from PCR, for amplifying such RNAs to detectable levels.

Since RNA does not serve as a template for PCR, it must first be copied into cDNA by a reverse transcriptase (Figure 5.1.1). Then the cDNA can be amplified by a standard PCR enzyme to a detectable level. This powerful coupling of reverse transcription (RT) with PCR has become a standard laboratory procedure.

This section describes several factors that can dramatically affect the success of an RT-PCR procedure. This information will be especially useful to those who need to design an RT-PCR system.



**Figure 5.1.1. Principle of RT-PCR.** A reverse transcriptase is used for primer-directed transcription of RNA into first-strand cDNA. Then, a DNA polymerase is used to amplify the cDNA. The RT-PCR process can be either a one-step process (*i.e.*, reverse transcription and amplification, performed consecutively in one tube) or a two-step process (reverse transcription and amplification performed in separate tubes).

### 5.1.1 Choosing a One-Step or a Two-Step Procedure

Reverse transcription and subsequent PCR can be performed as either a two-step procedure (with RT and PCR performed in separate tubes) or as a coupled, one-step procedure (with the RT and PCR performed consecutively in a single tube). Each procedure has its advantages:

Advantages of One-Step Procedure	Advantages of Two-Step Procedure
<b>Minimizes time required</b> <ul style="list-style-type: none"><li>▶ Has fewer pipetting steps than the two-step procedure.</li><li>▶ Significantly reduces the total time required for RT-PCR.</li></ul>	<b>Allows optimal reaction conditions</b> <ul style="list-style-type: none"><li>▶ Both RT and PCR can be performed under optimal conditions to ensure efficient and accurate amplification.</li></ul>
<b>Reduces risk of contamination</b> <ul style="list-style-type: none"><li>▶ Entire reaction takes place in a single tube.</li><li>▶ Requires no transfers and no opening of the tube at intermediate stages.</li><li>▶ Procedure can be automated.</li></ul>	<b>Provides maximum flexibility</b> <ul style="list-style-type: none"><li>▶ Allows choice of primers [random hexamer primers, oligo(dT), anchored oligo(dT) or sequence-specific primers].</li><li>▶ cDNA from a single reverse transcription can be used in several PCRs, allowing analysis of multiple transcripts.</li><li>▶ Allows a wider choice of RT and PCR enzymes</li></ul>
<b>Improves sensitivity and specificity</b> <ul style="list-style-type: none"><li>▶ Use of sequence-specific primers enhances specificity and sensitivity.</li><li>▶ Entire cDNA sample is used as template for the PCR.</li></ul>	<b>Amplifies long sequences</b> <ul style="list-style-type: none"><li>▶ With the right combination of enzymes, two-step RT-PCR can amplify RNA sequences up to 14 kb long.</li></ul>

# 5

### 5.1.2 Choosing the RT-PCR Enzymes

Obviously, a major factor to consider is the choice of reverse transcriptase used to synthesize cDNA. Since each of the available enzymes has different enzymatic properties, one may be more suitable for a specific experiment than the others. Among the enzyme properties to consider are:

- ▶ **Maximum length of template that can be transcribed into full-length cDNA**  
Some reverse transcriptases (RTases) can only transcribe short templates (less than 3 kb). Others can transcribe much longer templates (up to 14 kb). Be sure to select an enzyme that is likely to produce a full-length cDNA from your chosen RNA target.
- ▶ **Temperature optimum**  
Higher incubation temperatures help eliminate problems of template secondary structure and decrease false priming from sequence-specific primers. Thus, RTases that can be incubated at high temperatures are more likely to produce accurate copies of the RNA target, especially if it has a high GC content.
- ▶ **Sensitivity**  
RTases differ in their ability to copy small amounts of template. This is especially important when the chosen target is expressed at a very low level.
- ▶ **Specificity**  
RTases also differ in their ability to prepare accurate, full-length cDNA copies from difficult templates (*e.g.*, those with large amounts of secondary structure).

► Incorporation of modified nucleotides

If the cDNA is to be used in microarray analysis, the RTase must be able to incorporate several modified nucleotides (e.g., Cy3-, Cy5-, aminoallyl-, DIG- and biotin-dUTP).

► RNase H activity

RNase H removes the RNA from the RNA:cDNA hybrid. This can increase the sensitivity of the subsequent PCR. However, unless carefully controlled, RNase H activity may compete with cDNA synthesis, resulting in loss of template before full-length cDNA is transcribed. RTases have differing amounts of RNase H activity; some enzymes lack this activity.

Use the tables on the following pages to determine which RTase is best for your experimental system.

### Properties of Reverse Transcriptases Available from Roche Applied Science

Enzyme	Maximum Length of Template (kb)	Temperature Optimum	Sensitivity	Transcribes Difficult Templates	Makes Full-Length cDNA	RNase H Activity	Incorporates Modified Nucleotides	Comments
Transcriptor Reverse Transcriptase	14	42 to 65°C	+++	+++	+++	Yes	Yes	<ul style="list-style-type: none"> <li>► Component of Transcriptor First Strand cDNA Synthesis Kit (for Two-step RT-PCR). (See protocol D on page 147)</li> <li>► Transcribes long, rare or difficult targets (See protocol E on page 149) and shows excellent performance in two-step qRT-PCR.</li> </ul>
Expand Reverse Transcriptase <sup>a</sup>	14	42 to 50°C	++	+	+++	No	Yes	<ul style="list-style-type: none"> <li>► Transcribes templates with normal amounts of secondary structure.</li> <li>► For Two-step RT-PCR (See protocol F on page 151)</li> </ul>
AMV Reverse Transcriptase	12	42°C (up to 60°C)	++	+	++	Yes	Yes	<ul style="list-style-type: none"> <li>► Component of First Strand cDNA Synthesis Kit for RT-PCR (AMV) (for Two-step RT-PCR).</li> <li>► Component of Titan One Tube RT-PCR System and Kit (for One-step RT-PCR). (See protocols A, page 137, and B, page 140)</li> </ul>
M-MuLV Reverse Transcriptase	10	37°C	+	+(+)	++	Yes	Yes	<ul style="list-style-type: none"> <li>► Transcribes abundant, simple RNA targets.</li> </ul>
<i>C. therm.</i> Polymerase	3	60 to 70°C	++	+++	+	No	Yes	<ul style="list-style-type: none"> <li>► Component of <i>C. therm.</i> Polymerase One-Step RT-PCR System (for one-step RT-PCR). (See protocol C on page 140)</li> </ul>
Tth DNA Polymerase	1	55 to 70°C	+	+	-	No	Yes	<ul style="list-style-type: none"> <li>► Uses Mn<sup>2+</sup> as divalent cation, which can lower the fidelity of transcription.</li> <li>► For One-step RT-PCR of short targets.</li> </ul>

a) Not for sale in the United States.

### RT-PCR Kits and Protocols

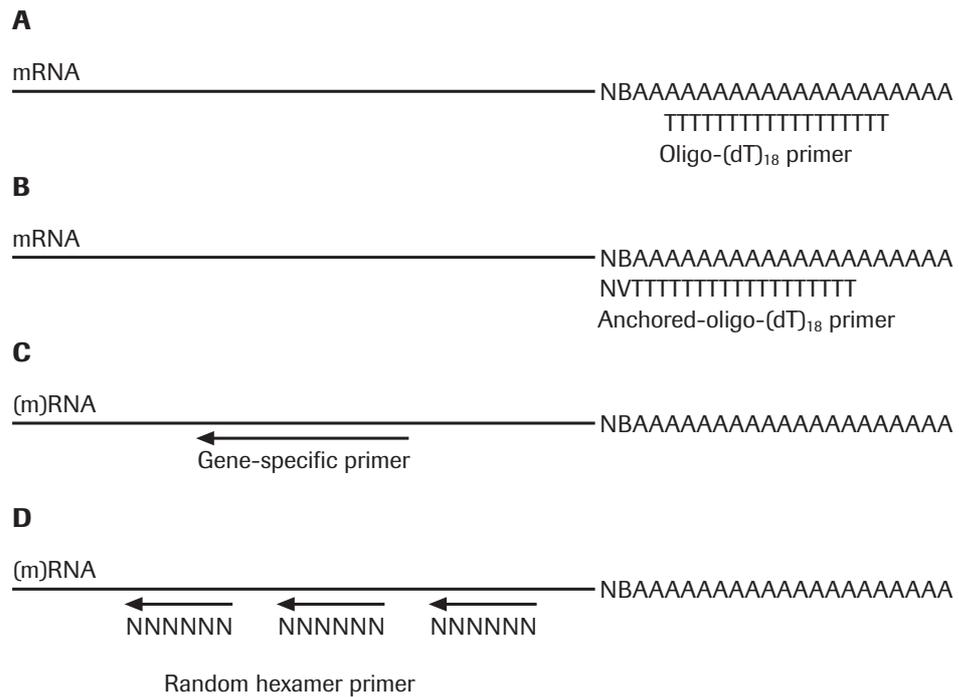
Application	Secondary Structure in Template	Maximum Length of Template	Suitable Kits from Roche Applied Science	For Details, See This Protocol in Chapter 5
One-Step RT-PCR	Normal	3 kb 6 kb	Titan One Tube RT-PCR System Titan One Tube RT-PCR Kit	One-Step RT-PCR with the Titan One Tube RT-PCR Kit Protocol A, page 137, protocol B, page 140)
	High	3 kb	<i>C. therm.</i> Polymerase One-Step RT-PCR System	One-Step RT-PCR with <i>C. therm.</i> Polymerase One-Step RT-PCR System Protocol C, page 140
Two-Step RT-PCR	Normal	12 kb	First Strand cDNA Synthesis Kit for RT-PCR (AMV)	–
	Normal	12 kb	cDNA Synthesis System	–
	Normal or High	12 kb	Microarray cDNA Labeling Kit Microarray cDNA Synthesis Kit	–
	High	14 kb	Transcriptor First Strand cDNA Synthesis Kit	Two-Step RT-PCR Protocol D, page 147 and protocol E, 149)

### 5.1.3 Choosing the Primers for Reverse Transcription

The primer used for reverse transcription affects both the size and the specificity of the cDNA produced. Four kinds of primers are commonly used in RT-PCR (Figure 5.1.2) and each has its advantages:

Type of RT Primer	Binds	Advantages/Comments
Oligo(dT) <sub>N</sub>	Endogenous poly(A) tail found at the 3' end of eukaryotic ( <i>e.g.</i> , mammalian) mRNAs.	<ul style="list-style-type: none"> <li>▶ Generates full-length cDNAs.</li> <li>▶ If template contains oligo(A) stretches, the primer may bind these and lead to mispriming.</li> </ul>
Anchored oligo(dT) <sub>N</sub>	Very beginning of the poly(A) tail.	<ul style="list-style-type: none"> <li>▶ Prevents priming from internal sites of the poly(A) tail.</li> <li>▶ Generates full-length cDNA.</li> <li>▶ Preferred priming method for most two-step RT-PCR applications.</li> <li>▶ Available only as part of the Transcriptor First Strand cDNA Synthesis Kit.</li> </ul>
Random hexamers	Many sites throughout the length of an RNA	<ul style="list-style-type: none"> <li>▶ Provides uniform representation of all RNA sequences in mRNA.</li> <li>▶ Can prime cDNA transcription from RNAs that don't contain a poly(A) tail.</li> <li>▶ Adjusting the ratio of random primers to RNA in the RT reaction controls the average length of cDNAs formed. Example: A high ratio will generate relatively short cDNAs, which will increase the chances of copying the complete target sequence.</li> <li>▶ Short cDNA transcripts may be ideal way to overcome difficulties presented by RNA secondary structures.</li> </ul>
Sequence-specific	Only sequences that are exactly complementary to the primer sequence	<ul style="list-style-type: none"> <li>▶ Selects for a particular RNA, <i>e.g.</i>, for diagnostic purposes.</li> <li>▶ Greatly increases the specificity of the RT-PCR.</li> <li>▶ Only type of priming that can be used for one-step applications.</li> </ul>

# 5



**Figure 5.1.2. Overview of first-strand cDNA synthesis with different types of primers.** **Panel A**, oligo(dT)<sub>n</sub> primer (in this case n = 18). **Panel B**, anchored oligo(dT)<sub>n</sub> primer (in this case n = 18). Starts at the very beginning of the poly(A) tail. **Panel C**, sequence-specific (gene-specific) primer. **Panel D**, random hexamers. V = A, C or G. B = C, G or T. N = A, C, G or T.

### Designing Sequence-Specific Primers

RT-PCR amplification of a particular RNA sequence requires two PCR primers that are specific for the sequence. The primer design should allow differentiation between the amplified product from cDNA and an amplified product derived from contaminating genomic DNA. There are two approaches to designing the required primers (Figure 5.1.3):

1. Make primers that anneal to sequences in exons on both sides of an intron (figure, panel 1). With such primers, any product amplified from genomic DNA will be much larger than a product amplified from intronless cDNA.
2. Make primers that span exon/exon boundaries on the RNA (figure, panel 2). Such primers should not amplify genomic DNA.

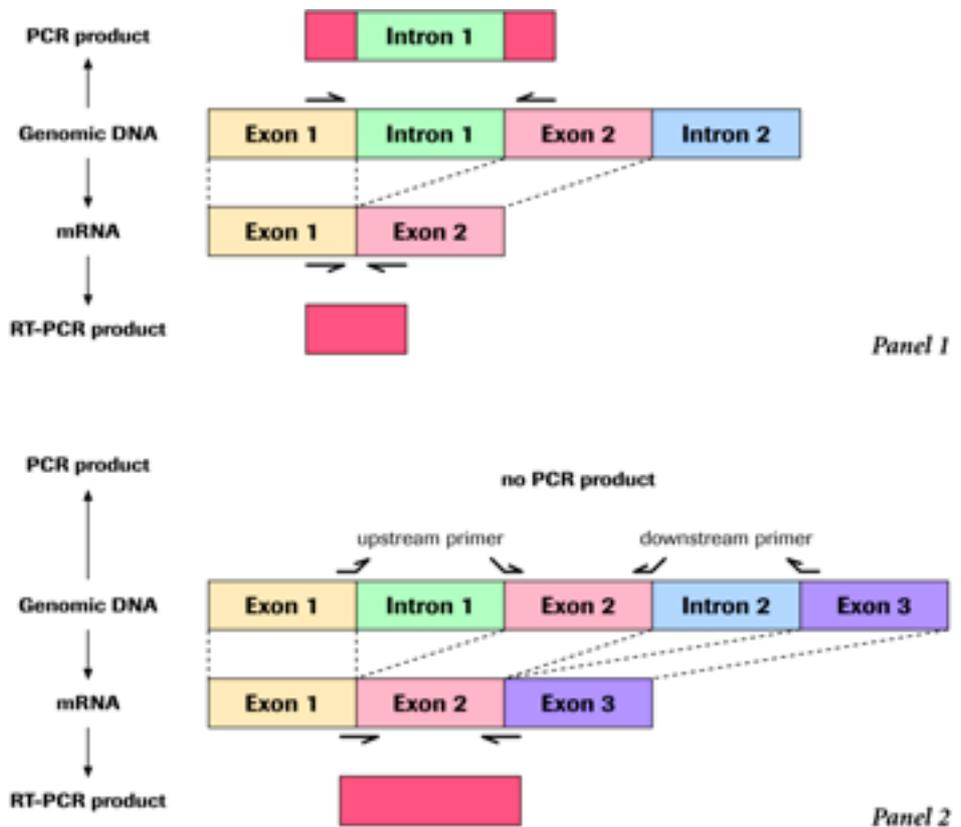


Figure 5.1.3. Two approaches to primer design. For explanation of panels, see text.

### 5.1.4 Preparation and Handling of Template RNA

Successful RT-PCR requires a high quality, intact RNA template. Use the recommendations in the following table to ensure that your RNA is both high quality and intact:

Area of Concern	Recommendations
Purity of template	<p><b>Start with the purest RNA template possible</b></p> <ul style="list-style-type: none"> <li>▶ If your target is mRNA, start with purified mRNA as template, rather than total RNA. (The proportion of mRNA in a total RNA preparation is quite low.) This will greatly increase the likelihood of successfully amplifying rare mRNAs.</li> <li>▶ Use a template preparation product that is specifically designed for RNA purification. For details on RNA purification products available from Roche Applied Sciences, see Chapter 3 of this manual.</li> <li>▶ If your target is mRNA, check the integrity of the mRNA by gel electrophoresis. The mRNA should appear as a smear between 500 bp and 8 kb. Most of the mRNA should be between 1.5 kb and 2 kb. If your target is eukaryotic total RNA, check the integrity of the total RNA by gel electrophoresis. The total RNA is dominated by ribosomal RNA, so the 18S and 28S rRNA should be appear as clear bands.</li> </ul>
Quality of template	<p><b>Eliminate RT inhibitors from the template preparation.</b></p> <ul style="list-style-type: none"> <li>▶ Before using it as a template, precipitate the RNA using ethanol, then wash it once with 70% ethanol. Be sure to remove all traces of ethanol before using the RNA in the RT reaction.</li> <li>▶ Use a product specifically designed for RNA purification to prepare the template. Such products can help eliminate potential inhibitors during purification. For details on RNA purification products available from Roche Applied Sciences, see Chapter 3 of this manual.</li> </ul>
Intactness of template	<p><b>Take rigorous and repeated precautions to prevent RNase contamination</b></p>
▶ during cell lysis	<ul style="list-style-type: none"> <li>▶ Use isolation methods that simultaneously disrupt cells and inactivate RNases (by adding SDS or guanidine). (See, for example, Chomczynski and Sacchi, 1987.)</li> </ul>
▶ during the purification procedure	<ul style="list-style-type: none"> <li>▶ Even if you don't think RNases could be present at a particular step, act as though they are present.</li> <li>▶ Include RNase inhibitors (<i>e.g.</i>, Protector RNase Inhibitor*) in non-denaturing purification steps. Example: Add Protector RNase Inhibitor to the water you use for resuspending the isolated RNA.</li> <li>▶ Use only sterile, RNase- and DNase-free plastic disposables (pipette tips, etc.).</li> <li>▶ Sterilize all glassware before use. (RNases can be present on non-sterile glassware.).</li> <li>▶ Always wear gloves throughout the isolation process. (Skin is a rich source of RNases.).</li> <li>▶ If necessary, analyze the product of each step in the isolation process by gel electrophoresis to ensure that the RNA is still RNase-free.</li> </ul>
▶ after purification	<ul style="list-style-type: none"> <li>▶ Short-term storage: Store the purified RNA template at +2 to +8°C until use.</li> <li>▶ Long-term storage: Store the purified RNA template at -70°C.  If possible, store cDNA, it is more stable than RNA.</li> </ul>

\* Available from Roche Applied Science.

## 5.2 Using Protector RNase Inhibitor

Protector RNase Inhibitor inactivates a wide spectrum of common RNases. For example, a 1 U/ $\mu$ l concentration of RNase Inhibitor, when added to a 20  $\mu$ l sample of MS2 RNA, completely protects the RNA from degradation by the following RNases:

- ▶ Up to 1 ng RNase A
- ▶ Up to 160 pg RNase B
- ▶ Up to 0.6 U RNase T2

Unlike other RNase inhibitors, Protector RNase Inhibitor is effective in a wide variety of applications, because it:

- ▶ Is active over a wide pH range (5.0 to 9.0)
- ▶ Is active over a wide temperature range (25 to 55°C, with some activity even at 60°C)



*This thermal stability is particularly important, since many RNase inhibitors are inactive at temperatures above 50°C and can't protect the RNA during cDNA synthesis.*

- ▶ Does not interfere with enzymes commonly used to modify or assay RNA (so it will inhibit RNases rather than your assay).
- ▶ Is produced under stringent GMP conditions.

### 5.2.1 How To Use Protector RNase Inhibitor

Use the amount of Protector RNase Inhibitor listed in the table below to protect against RNase degradation occurring during the following applications:

Application	Protector RNase Inhibitor (Working Concentration)
One-step RT-PCR	5–10 U/reaction
cDNA synthesis (for two-step RT-PCR)	25–50 U/reaction
<i>In vitro</i> transcription/translation	20 U/reaction



*You may use higher concentrations of Protector RNase Inhibitor in RT-PCR if you suspect that high levels of RNase contamination make certain samples difficult to amplify. The inhibitor will not interfere with RT-PCR, even when added at 16-fold higher concentrations than those listed above.*

## 5.3 One-Step RT-PCR

### Purpose of Procedure:

Reverse transcription and specific amplification of normal or GC-rich RNA targets (up to 6 kb) in a single tube.

### Enzyme/System Needed:

Titan One Tube RT-PCR Kit or System (for RNAs up to 6 kb)

*C. therm* Polymerase One-Step RT-PCR System (for RNAs up to 3 kb)



For more information on choosing the correct RT-PCR procedure, see “Factors To Consider in RT-PCR” at the beginning of Chapter 5.

### 5.3.1 Reagents and Equipment Required

One-step RT-PCR involves performing reverse transcription and PCR amplification consecutively in a single tube. For best results, use the following guidelines for choosing the optimal one-step RT-PCR kit:

- ▶ For high sensitivity RT-PCR of normal or RNA targets (up to 6 kb) in a single tube, choose either the Titan One Tube RT-PCR Kit or the Titan One Tube RT-PCR System.
- ▶ For RT-PCR of RNA targets (up to 3 kb) that have extensive secondary structure (e.g., GC-rich mRNA or viral RNA), choose the *C. therm* Polymerase One-Step RT-PCR System.



For details on the characteristics of these kits, see “Choosing the RT-PCR Enzymes” in section 5.1. For pack sizes, see Ordering Information in the Appendix.

### Additional Equipment and Reagents Required

The additional equipment and reagents needed will depend on the exact RT-PCR kit used. For details, see the individual protocols in section 5.3.3 below.

### 5.3.2 General Considerations for One-Step RT-PCR

#### Sample Material

High quality intact RNA is essential for good results. Be especially careful to avoid contaminating the RNA with RNases at any step in the isolation process. Always follow the precautions listed under “Preparation and Handling of Template RNA” in section 5.1 (“Factors To Consider in RT-PCR”). For more information on preparing high quality RNA templates, see Chapter 3.

The amount of template needed will depend greatly on the source of the RNA and the design of the experiment. Therefore, the optimal template concentration should always be determined empirically. For initial experiments, try:

RNA Template	Initial Conc. for Titan Enzyme	Initial Conc. for <i>C. therm</i> Enzyme
Total RNA	1 pg - 1 μg	50 pg - 1 μg



*Both the Titan and the C. therm protocols (given in section 5.3.3 below) are very sensitive. Under optimal conditions, the Titan protocol can generate detectable PCR products from as few as 1000 copies of an RNA target. Similarly, the C. therm protocol can generate detectable PCR products from as little as 100 pg total RNA or target concentrations as low as 100 copies/μl.*

#### PCR Primers

For one-step RT-PCR, always use sequence-specific PCR primers. Make sure the primers are not self-complementary.



*For information on choosing primers, see “Choosing the Primers for Reverse Transcription” in section 5.1 (“Factors To Consider in RT-PCR”).*

### 5.3.3 Protocols for One-Step RT-PCR

#### Protocol A: Standard One-Step RT-PCR with the Titan One Tube RT-PCR Kit (for RNA templates up to 3 kb)

##### About the Components of the Titan One Tube Kit and the Titan One Tube System

The Titan One Tube RT-PCR System and Kit both contain:

- ▶ RT-PCR enzyme mix containing AMV reverse transcriptase (for reverse transcription) and the Expand High Fidelity PCR System (enzyme blend of Taq DNA Polymerase and a proofreading polymerase, for PCR).
- ▶ Optimized RT-PCR buffer, MgCl<sub>2</sub> and DTT.

For optimal convenience, the Titan Kit contains additional reagents not supplied in the Titan System:

- ▶ Additional reaction components for quick, convenient reaction set-up (PCR Grade Nucleotide Mix, PCR Grade Water, and RNase inhibitor).
- ▶ Positive controls (human control RNA and primer mix for amplification of a 587-base sequence from a beta-actin mRNA target).

##### Additional Equipment and Reagents Required



*The protocol below uses the Titan One Tube RT-PCR Kit. The protocol may also be performed with the Titan One Tube RT-PCR System (rather than the Titan Kit) if you purchase the additional reagents required (as listed below).*

If you are using the Titan One Tube RT-PCR Kit, you will need:

- ▶ Thermal block cycler
- ▶ 0.2 ml thin-walled PCR tubes\*
- ▶ Sterile reaction tubes for preparing master mixes and dilutions

If you are using the Titan System rather than the Titan Kit, you will also need these additional reagents:

- ▶ Protector RNase Inhibitor\*
- ▶ PCR Grade Nucleotide Mix (10 mM of each dNTP)\*
- ▶ PCR Grade Water\*

\* available from Roche Applied Science; see Ordering Information (in the Appendix) for details.



*Before setting up the reactions, program the thermal block cycler for the amplification conditions listed under "Standard RT-PCR" below. Preheat the block cycler to the temperature of the RT reaction (50 to 60°C).*

### Setting Up the Reaction

- 1
  - ▶ Thaw all frozen reagents before use.
  - ▶ Mix all reagents carefully (by pipetting up and down) and briefly centrifuge them before starting the procedure.
  - ▶ Store the reagents on ice while setting up the reaction.
- 2 Prepare two separate master mixes in sterile, nuclease-free 1.5 ml microfuge tubes (on ice). (Master Mix 1 contains dNTPs, primers and template DNA; Master Mix 2 contains RT-PCR buffer and enzyme mix.)
  - ! For each mix, add the components in the order listed below. Always wear gloves when handling RNA. (Numbered vials are supplied in the kit.)

#### Master Mix 1 (for one reaction)

Components (Mix 1) <sup>a</sup>	Volume	Final conc.
PCR Grade Water (vial 6 from kit)	variable	to make total vol = 25 µl
PCR Nucleotide Mix, 10 mM (vial 2)	4 µl	200 µM (each nucleotide)
DTT solution (supplied in kit)	2.5 µl	5 mM
RNase Inhibitor (vial 3) <sup>b</sup>	1 µl	5 U/reaction
Downstream primer <sup>c</sup>	variable	0.4 µM
Upstream primer <sup>c</sup>	same as downstream primer	0.4 µM
Template RNA (isolated total RNA) <sup>c</sup>	variable	1 pg–1 µg total RNA
<b>Final volume (Mix 1)</b>	<b>25 µl</b>	

<sup>a</sup>) To prepare Master Mix 1 for more than one reaction, multiply the amounts of all components (except template RNA) by Z, where Z = the number of reactions to be run + one additional reaction. Mix these components to form a homogenous solution, aliquot equal amounts of the solution to new microfuge tubes (one for each reaction, on ice), and add template RNA to each..

<sup>b</sup>) Protector RNase Inhibitor is more concentrated (40 U/µl) than the RNase Inhibitor supplied with the kit. If you are using Protector RNase Inhibitor, add 0.5 µl Protector RNase Inhibitor (20 U) to each reaction and adjust the water in the tube accordingly.

<sup>c</sup>) The kit contains a control RNA and primers. To make a positive control reaction, use 2 µl of the β-actin control primer mix (vial 5 of the kit) and 5 µl of the human control RNA (vial 4, 2 pg/µl). The correct amount of water to add to this control reaction is 10.5 µl.

#### Master Mix 2 (for one reaction)

Components (Mix 2) <sup>d</sup>	Volume	Final conc.
PCR Grade water (vial 6)	14 µl	
RT-PCR buffer with 7.5 mM MgCl <sub>2</sub> , 5× conc. (supplied with kit)	10 µl	1.5 mM MgCl <sub>2</sub>
Titan enzyme mix (vial 1)	1 µl	
<b>Final volume (Mix 2)</b>	<b>25 µl</b>	

<sup>d</sup>) To prepare Master Mix 2 for more than one reaction, multiply the amounts of all components by Z, where Z = the number of reactions to be run + one reaction.

- 3 Mix the reagents in each Master Mix carefully and centrifuge briefly.
  - ! Do not vortex!



- 4** ▶ For each reaction, combine 25 µl Master Mix 1 (including template) and 25 µl Master Mix 2 in a thin-walled PCR tube on ice.  
 ▶ Gently mix the solutions to produce a homogeneous reaction mixture.  
 ▶ According to the instructions supplied with your thermal block cycler, prepare the tubes for cycling (e.g., overlay the reaction with mineral oil if necessary).

- 5** Place PCR tubes in the preheated thermal block cycler and begin RT-PCR immediately.

 *The completed reaction mixtures should not be stored for extended periods of time.*

### Standard RT-PCR

After placing the samples in the thermal block cycler, cycle according to the thermal profile below.

 *The RNA template does not need to be denatured. Do not include an RNA denaturation step before the RT reaction.*

*The profile below was developed for the Applied Biosystems GeneAmp PCR System 2400 thermal block cycler. Other PCR instruments may require a different profile.*

Cycles		Temperature	Time
<b>Reverse Transcription</b>			
1		50 to 60°C <sup>a</sup>	30 min
<b>Initial Denaturation</b>			
1		94°C	2 min
<b>Amplification Program 1</b>			
10	Denaturation	94°C	30 s
	Annealing	45 to 66°C <sup>b</sup>	30 s
	Elongation	68°C	45 s to 2 min <sup>c</sup>
<b>Amplification Program 2</b>			
25 <sup>d</sup>	Denaturation	94°C	30 s
	Annealing	45 to 66°C <sup>b</sup>	30 s
	Elongation	68°C	(45 s to 2 min) <sup>c</sup> + 5 s cycle elongation for each successive cycle <sup>e</sup>
<b>Final Elongation</b>			
1		68°C	7 min

<sup>a)</sup> For initial experiments, we recommend 50°C. If you perform the RT reaction at a temperature well above 50°C, the AMV reverse transcriptase will be partially inactivated and will produce only short (<1kb) cDNA fragments.

<sup>b)</sup> Optimal annealing temperature depends on the melting temperature of the primers and the system used. Use 66°C for the human β-actin control primers (positive control supplied in kit).

<sup>c)</sup> Elongation time depends on length of the amplicon. Use the following guidelines: 45 s for <1 kb, 1.5 min for 1–1.5 kb, 2 min for 3 kb.

<sup>d)</sup> A total of 35 PCR cycles is usually enough to amplify the cDNA. However, if the target mRNA is rare, increase the number of cycles in Amplification Program 2.

<sup>e)</sup> In this part of the program, each cycle will be 5 s longer than the previous cycle. Gradually increasing extension time ensures a higher yield of amplification products.

### Protocol B: One-Step RT-PCR with the Titan One Tube RT-PCR Kit for Long (up to 6 kb) Templates

Use the same reaction set-up as in Protocol A above. Use the amplification conditions listed under “Standard RT-PCR” above, except lower the temperature for reverse transcription and increase the elongation time in amplification programs 1 and 2, according to the following guidelines:

PCR target length (kb)	3	4.5	6
Temperature for RT step (°C)	50	48	45 to 48
Elongation time	2 min	3 min	4 min

### Protocol C: One Step RT-PCR for GC-rich Templates (up to 3 kb) with the *C. therm.* Polymerase One Step RT-PCR System

#### About the *C. therm.* Polymerase One-Step RT-PCR System

Because the *C. therm.* enzyme can perform the RT reaction at elevated temperatures (between 60 and 70°C), the *C. therm.* Polymerase One-Step RT-PCR System is particularly suitable for amplifying templates with troublesome secondary structures. At these elevated temperatures, many secondary structures “melt out,” so the specificity of primer binding is enhanced.

Performance-enhancing components of the *C. therm.* Polymerase One-Step RT-PCR System include:

- ▶ *C. therm.* “reverse transcriptase,” which is the Klenow fragment of the thermostable DNA polymerase from *Carboxydotherrnus hydrogenoformans*; this enzyme can reverse transcribe RNAs up to 3 kb long.
- ▶ Taq DNA Polymerase, which can amplify cDNAs up to 3 kb long.
- ▶ Dimethylsulfoxide (DMSO) in the RT-PCR buffer, which reduces the stability of secondary structures such as hairpins.

#### Additional Equipment and Reagents Required

- ▶ Thermal block cycler
- ▶ 0.2 ml thin-walled PCR tubes\*
- ▶ Sterile reaction tubes for preparing master mixes and dilutions
- ▶ Protector RNase Inhibitor\*
- ▶ PCR Nucleotide Mix, 10 mM (each nucleotide)\*
- ▶ PCR-Grade Water\*

\* available from Roche Applied Science; see Ordering Information (in the Appendix) for details.

## Setting Up the Reaction

 Before setting up the reactions, program the thermal block cycler for the RT-PCR conditions listed under “RT-PCR” below. Preheat the thermal block cycler to the temperature of the RT reaction (60 to 70°C).

### Setting Up the Reaction

- 1  Thaw all frozen reagents before use.
-  Mix all reagents carefully (by pipetting up and down) and briefly centrifuge them before starting the procedure.
-  Keep the reagents on ice while setting up the reaction.

- 2 Prepare two separate master mixes in sterile, nuclease-free 1.5 ml microfuge tubes (on ice). (Master Mix 1 contains dNTPs, primers, and template DNA; Master Mix 2 contains RT-PCR buffer and enzyme mix.)

 For each mix, add the components in the order listed below. Always wear gloves when handling RNA. (Numbered vials are supplied with the kit.)

#### Master Mix 1 (for one reaction)

Components (Mix 1) <sup>a</sup>	Volume	Final conc.
PCR Grade Water	variable	to make total vol = 25 µl
PCR Nucleotide Mix, 10 mM	2 µl	400 µM (each nucleotide)
DMSO (vial 3) <sup>b</sup>	2.5 µl	7% (including DMSO in reaction buffer)
DTT solution (vial 4)	2.5 µl	5 mM
Protector RNase Inhibitor	0.5 µl	20 U/reaction
Downstream primer	variable	0.3 µM
Upstream primer	same as downstream primer	0.3 µM
Template RNA (isolated total RNA)	variable	50 pg–1 µg total RNA
<b>Final volume (Mix 1)</b>	<b>25 µl</b>	

<sup>a)</sup> To prepare Master Mix 1 for more than one reaction, multiply the amounts of all components (except template RNA) by Z, where Z = the number of reactions to be run + one additional reaction. Mix these components to form a homogenous solution, aliquot equal amounts of the solution to new microfuge tubes (one for each reaction, on ice), and add template RNA to each.

<sup>b)</sup> If you detect secondary products in initial experiments or if you get very little full-length product, repeat this protocol with an increased amount of DMSO (up to a maximum of 10%).

#### Master Mix 2 (for one reaction)

Components (Mix 2) <sup>b</sup>	Volume	Final conc.
PCR Grade Water	13 µl	
RT-PCR buffer with 10% DMSO and 12.5 mM MgCl <sub>2</sub> , 5× conc. (vial 2)	10 µl	2.5 mM MgCl <sub>2</sub>
C. therm. enzyme mix (vial 1)	2 µl	
<b>Final volume (Mix 2)</b>	<b>25 µl</b>	

<sup>b)</sup> To prepare Master Mix 2 for more than one reaction, multiply the amounts of all components by Z, where Z = the number of reactions to be run + one reaction.



- 3 Mix the reagents in each tube carefully and centrifuge briefly.
  - 4
    - ▶ For each reaction, combine 25  $\mu$ l Mix 1 (including template RNA) and 25  $\mu$ l Mix 2 in a thin-walled PCR tube on ice.
    - ▶ Gently mix the solutions to produce a homogeneous reaction mixture.
    - ▶ According to the instructions supplied with your instrument, prepare the tubes for PCR (e.g., overlay reaction mixture with mineral oil if necessary).
  - 5 Place PCR tubes in the preheated thermal block cycler and begin RT-PCR immediately.
-  *The completed reaction mixtures should not be stored for extended periods of time.*

### RT-PCR

After placing samples in the thermal block cycler, cycle according to the thermal profile below.

-  *The RNA template does not need to be denatured. Do not include an RNA denaturation step before the RT reaction.*  
*The profile below was developed for the Applied Biosystems GeneAmp PCR System 2400 thermal block cycler. Other PCR instruments may require a different profile.*

Cycles		Temperature	Time
<b>Reverse Transcription</b>			
1		60 to 70°C <sup>a</sup>	30 min
<b>Initial Denaturation</b>			
1		94°C	2 min
<b>Amplification Program 1</b>			
10	Denaturation	94°C	30 s
	Annealing	60 to 70°C <sup>a</sup>	30 s
	Elongation	70 to 72°C	45 s to 3 min <sup>b</sup>
<b>Amplification Program 2</b>			
20–30 <sup>c</sup>	Denaturation	94°C	30 s
	Annealing	60 to 70°C <sup>a</sup>	30 s
	Elongation	70 to 72°C	60 s/kb <sup>b</sup> + 5 s cycle elongation for each successive cycle <sup>d</sup>
<b>Final Elongation</b>			
1		70 to 72°C	7 min

<sup>a)</sup> For initial experiments, use 60°C. If results are unsatisfactory, the temperature may be increased (to a maximum of 70°C) to eliminate secondary RNA structures. Be sure the RT primer will still bind at these elevated temperatures.

<sup>b)</sup> Elongation time depends on product length. Allow approx. 1 min amplification per kb product length.

<sup>c)</sup> The number of cycles required depends on the abundance of the target mRNA. For rare mRNA messages, 40 cycles (total) may be required.

<sup>d)</sup> Gradually increasing extension time ensures a higher yield of amplification products. For example, cycle no. 11 is 5 s longer than cycle 10, cycle no. 12 is 10 s longer than cycle 10, cycle no. 13 is 15 s longer than cycle 10, etc.

## Troubleshooting the RT-PCR

If initial results with any of the above protocols (A – C) are unsatisfactory, try the following:

- ▶ Increase the amount of RNA template (up to a total of 1 µg total RNA) or use purified mRNA as template.
- ▶ Titrate the concentration of PCR primers. Try varying the concentrations of each primer from 0.2 to 1.0 µM. (Always use equal concentrations of the forward primer and the reverse primer.)
- ▶ Use a lower temperature for the RT and/or the annealing steps.
- ▶ If the cDNA has a high GC content (>60%), increase the denaturation time.
- ▶ Repeat the RNA template isolation, being careful to avoid RNase contamination and reverse transcriptase inhibitors.



*For a more detailed list of options, see “Troubleshooting” in the Appendix.*

## Processing of PCR Products

### Analysis

Analyze the products on a 1 - 2% agarose gel.

### Storage

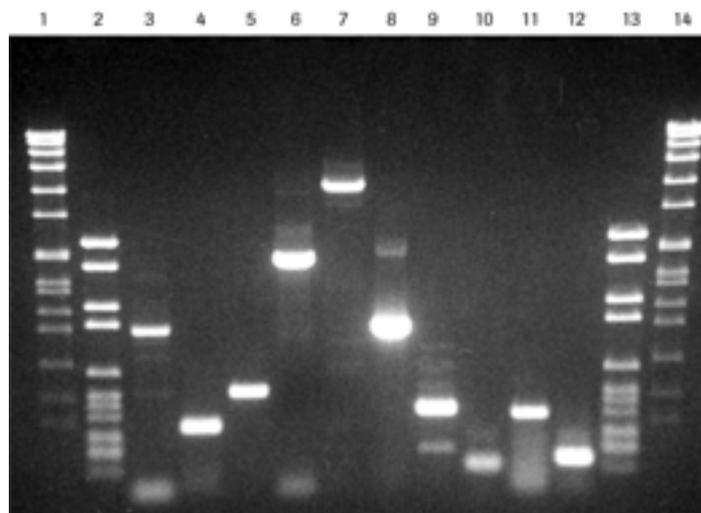
For short-term storage, cool the samples and store at 2 to 8°C for up to 2 h. For long-term storage, PCR products up to 4.5 kb may be stored frozen at -15 to -25°C.

### Cloning

We recommend TA cloning, since DNA products obtained from this protocol have a single A overhang (3' end).

### 5.3.4 Typical Results

**Result:** The Titan One Tube RT-PCR System and Kit allow specific one-step RT-PCR amplification of 0.15 – 4.0 kb RNA targets, even when the template is available in limited quantity. The yield from each of the reactions was excellent.

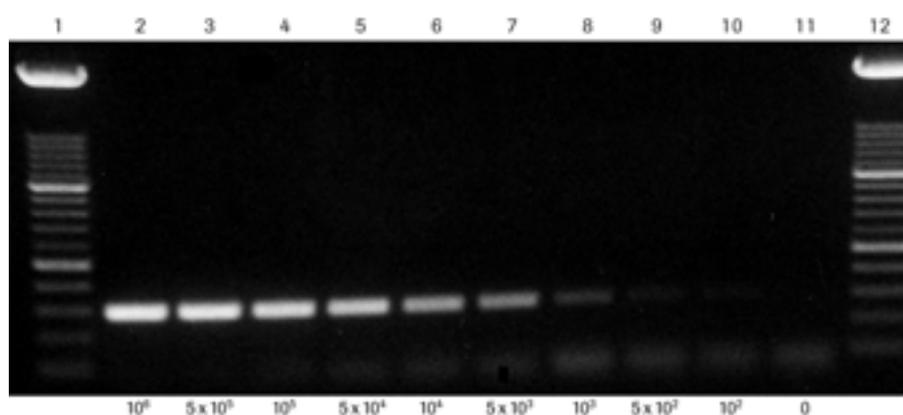


**Figure 5.3.1. Amplification of various RNA targets (0.2 – 4 kb) with the Titan One Tube RT-PCR System.** RT-PCR was performed with the Titan One Tube RT-PCR System according to protocol A above (cDNA synthesis at 50°C, 35 cycles of PCR). Various RNA templates and gene-specific primers were used. An aliquot (15 µl from the 50 µl reaction mixture) of each PCR product was analyzed on a 1.2% agarose gel. The various templates and products are listed below:

**Lanes 1, 14:** Molecular Weight Marker VII. **Lanes 2, 13:** Molecular Weight Marker VI.

**Lane 3:** 980 bp GAPDH (glyceraldehyde-3-phosphate dehydrogenase) product, amplified from 10 ng human liver RNA. **Lane 4:** 324 bp β-actin target, amplified from 1 ng total mouse liver RNA. **Lane 5:** 530 bp MCAD (medium-chain acetyl-CoA dehydrogenase) product, amplified from 10 ng total human liver RNA. **Lane 6:** 1.9 kb dystrophin target, amplified from 10 ng total human skeletal muscle RNA. **Lane 7:** 4.0 kb dystrophin target, amplified from 10 ng total human skeletal muscle RNA. **Lane 8:** 1 kb PMSA target, amplified from 100 ng total RNA from cell line LNCaP. **Lane 9:** 419 bp PMSA target, amplified from 100 ng total RNA from cell line LNCaP. **Lane 10:** 145 bp viral RNA target, amplified from approximately 5000 copies of message. **Lane 11:** 391 bp interleukin-1β target, amplified from 200 ng total RNA from human blood. **Lane 12:** 192 bp M-CSF (macrophage colony stimulating factor) target, amplified from 100 ng total human liver RNA.

**Result:** The *C. therm.* Polymerase One Step RT-PCR System can amplify viral RNA targets from templates that contain as few as 500 copies of viral RNA. (On the original gel, a faint 284 bp band can even be seen in the sample that contained just 100 copies of the viral RNA.)



**Figure 5.3.2. Amplification of viral RNA with the *C. therm.* Polymerase One-Step RT-PCR System.**

**Lanes 2-10:** Varying amounts of viral RNA (from  $10^6$  to  $10^2$  copies of the viral genome) were used as template. Sequence-specific primers were used to amplify a 284 bp target from the genome. One-step RT-PCR was performed as described in protocol C above (RT step, 30 min at 60°C; for PCR, primer annealing was at 60°C and elongation at 68°C). RT-PCR products were visualized (with ethidium bromide) on an agarose gel. The estimated number of copies of viral RNA in each sample is shown under the lanes of the gel. **Lane 1, 12:** molecular weight marker XIV. **Lane 11:** 0, negative control (no template).

## 5.4 Two-Step RT-PCR

### Purpose of Procedure:

Reverse transcription and specific amplification of both short and long RNA targets (up to 14 kb) in separate tubes, thus ensuring optimum conditions for each step.

### Enzyme/System Needed:

Many products are suitable. See details below.



*For more information on choosing the correct RT-PCR procedure, see “Factors To Consider in RT-PCR” at the beginning of Chapter 5.*

### 5.4.1 Reagents and Equipment Required

Two-step RT-PCR involves reverse transcription and PCR amplification in separate tubes. Especially with longer or problematic templates, choosing the right enzyme and reaction conditions for each step may be crucial for the success of the RT-PCR.

#### Enzyme or Kit for the Reverse Transcription Step

For amplification of RNA targets up to 14 kb long, the following kit and enzymes may be used for the first step (synthesis of first-strand cDNA by reverse transcription) of a two-step RT-PCR procedure:

- ▶ Transcriptor First Strand cDNA Synthesis Kit
- ▶ Transcriptor Reverse Transcriptase (Transcriptor RTase)
- ▶ Expand Reverse Transcriptase (Expand RTase)



*For details on the characteristics of these enzymes and kits, see “Choosing the RT-PCR Enzymes” in section 5.1. For pack sizes, see Ordering Information in the Appendix.*

#### Enzyme or Kit for the PCR Step

A wide variety of PCR enzymes are available from Roche Applied Science. For details, see Chapter 2. For more on selecting the correct enzyme, see section 5.4.4 below.

#### Additional Equipment and Reagents Required

The additional equipment and reagents needed will depend on the exact RT and PCR protocols chosen. For details, see the individual RT protocols in section 5.4.3 below and the individual PCR protocols in Chapter 4.



*We recommend using a thermal block cycler with a heated lid for all two-step RT-PCR procedures.*

## 5.4.2 General Considerations for Two-Step RT-PCR

### Sample Material

High quality intact RNA is essential for good results. Be especially careful to avoid contaminating the RNA with RNases at any step in the isolation process. For details on preventing RNase contamination, see “Preparation and Handling of Template RNA” in section 5.1 (“Factors To Consider in RT-PCR”). For more information on preparing high quality RNA templates, see Chapter 3.

For template RNA, use either 10 ng – 5 µg intact, total RNA, or 1 – 100 ng isolated, purified RNA *e.g.*, poly(A)<sup>+</sup> mRNA. If the RNA concentration in the template preparation is low, stabilize it by adding MS2 RNA\* (10 µg/ml) to the preparation.

For initial experiments try:

RNA Template	Initial Conc. for Transcriptor RTase	Initial Conc. for Expand RTase
Total RNA	1 µg	1 µg
mRNA <sup>a</sup>	10 ng	50 ng

a) To verify the integrity of an mRNA preparation, electrophorese the isolated mRNA on an agarose gel and stain the gel with ethidium bromide to determine the average size of the preparation. The mRNA should appear as a smear between approx. 500 bp and 8 kb. The bulk of the mRNA should be between 1.5 and 2 kb. If your target is eukaryotic total RNA, check the integrity of the total RNA by gel electrophoresis. The total RNA is dominated by ribosomal RNA, so the 18S and 28S rRNA should be appear as clear bands.

### Choice of Primers

Depending on the RNA to be amplified and the purpose of the amplification, choose one of the following types of primer:

▶ Oligo(dT)<sub>n</sub>\* is an oligomer (n usually between 15 and 20) that is mainly used to synthesize full-length cDNAs from poly(A)<sup>+</sup> mRNA.

▶ Anchored oligo(dT)<sub>18</sub> is a modified form of oligo(dT)<sub>n</sub> that binds at the very beginning of the poly(A) tail of mRNA to ensure generation of full-length cDNAs.



*Anchored oligo(dT)<sub>18</sub> is only available as part of the Transcriptor First Strand cDNA Synthesis Kit\*.*

▶ Random hexamers\* [p(dN)<sub>6</sub>] are short primers that often give uniform amplification of complex RNAs.



*You can adjust the ratio of random primers to RNA to control the average length of cDNA products. Higher ratios (e.g., 60 µM hexamer to 1 µg total RNA) produce relatively short cDNAs, while lower ratios (as low as 1.5 µM hexamer to 1 µg total RNA) generate longer cDNAs.*

▶ Sequence-specific primer will bind and specifically amplify a particular RNA or a related set of RNAs; this is the only primer that should be used for long (5 kb or more) targets.



*For more information on choosing primers, see “Choosing the Primers for Reverse Transcription” in section 5.1.*

\* Available from Roche Applied Science.

### 5.4.3 Protocols for the Reverse Transcription Step (Templates up to 14 kb)

#### Protocol D: Synthesis of First Strand cDNA with the Transcriptor First Strand cDNA Synthesis Kit

##### About the Transcriptor First Strand cDNA Synthesis Kit

The Transcriptor First Strand cDNA Synthesis Kit contains the following components:

- ▶ Transcriptor Reverse Transcriptase and optimized reaction buffer



*Transcriptor Reverse Transcriptase is a fast, thermostable reverse transcriptase that can complete first-strand cDNA synthesis in just 30 minutes and is ideal for GC-rich templates. For more information, see section below. It shows excellent performance in two-step qRT-PCR on different real-time instruments.*

- ▶ Protector RNase Inhibitor, a heat-stable, broad-spectrum RNase inhibitor



*For more information on Protector RNase Inhibitor, see the article on the inhibitor in this chapter.*

- ▶ A choice of two primers: anchored oligo(dT)<sub>18</sub> or random hexamers



*For more information, see above.*

- ▶ Deoxynucleotide Mix, 10 mM (each nucleotide)

- ▶ PCR Grade Water

- ▶ Positive Control Template and Sequence-Specific Control Primers



*For more information on using the positive control RNA and primers, see the package insert for the kit.*

##### Additional Equipment and Reagents Required

- ▶ Thermal block cycler with a heated lid.

## Setting Up and Running the RT Reaction with Transcriptor First Strand cDNA Synthesis Kit



Preheat the thermal block cycler to the temperature of the RT reaction (see step 6 below) before starting the procedure.

### Setting Up the Reaction

- 1
  - ▶ Thaw all frozen reagents before use.
  - ▶ Mix all reagents carefully (by pipetting up and down) and briefly centrifuge them before starting the procedure.
  - ▶ Keep all reagents on ice while setting up the reactions.
- 2 In a sterile, nuclease-free, thin-walled PCR tube (on ice), prepare the template-primer mixture for one 20 µl reaction by adding the components in the order listed below. Always wear gloves when handling RNA. (Numbered vials are supplied with the enzyme.)

#### Template-Primer Mix 1 (for one reaction)

Components	Volume	Final conc.
Total RNA or Poly(A) <sup>+</sup> mRNA	variable	1 µg total RNA or 10 ng poly(A) <sup>+</sup> RNA <sup>a</sup>
Primer – choose either:		
Anchored Oligo(dT) <sub>18</sub> , 50 pmol/ µl (vial 5)	1 µl	2.5 µM
or Random Hexamers, 600 pmol/ µl (vial 6)	2 µl	60 µM
or Sequence-Specific Primer	variable	0.5 – 2.5 µM
PCR Grade Water	variable	to make total volume = 13 µl
<b>Total volume</b>	<b>13 µl</b>	

<sup>a)</sup> These are the suggested concentrations for initial experiments. Suitable template concentrations may range from 10 ng to 5 µg total RNA and from 1 to 100 ng mRNA.

- 3 **Optional Step:** (only needed if template has extensive secondary structure, which should be heat denatured)
  - ▶ Denature the template-primer mixture by heating the tube for 10 min at 65°C in a thermal block cycler with a heated lid (to minimize evaporation).
  - ▶ Immediately cool the tube on ice.
- 4 To the tube containing the template-primer mix, add the rest of the components of the RT mix in the order listed below. (Numbered vials are supplied with the enzyme.)

#### Remainder of RT mix (for one reaction)

Components (Mix 2)	Volume	Final conc.
Transcriptor Reverse Transcriptase Reaction Buffer, 5× conc. (vial 2)	4 µl	8 mM MgCl <sub>2</sub>
Protector RNase Inhibitor, 40 U/µl (vial 3)	0.5 µl	20 U/reaction
Deoxynucleotide Mix, 10 mM each (vial 4)	2 µl	1 mM each
Transcriptor Reverse Transcriptase, 20 U/µl (vial 1)	0.5 µl	10 U/reaction
<b>Final volume (including template)</b>	<b>20 µl</b>	



- 5 ▶ Mix the reagents in the tube carefully.  
 *Do not vortex!*
- ▶ Centrifuge the tube briefly to collect the sample on the bottom of the tube.  
 ▶ Place the tube in a thermal block cycler with a heated lid (to minimize evaporation).

- 6 Depending on the primer used and the length of the target mRNA, incubate the RT reaction as described in the table below:

If you are using...	And the target mRNA is..	Incubate the RT reaction...
Anchored oligo(dT) <sub>18</sub> primer or Sequence-specific primer	up to 4 kb	30 min at 55°C
	>4 kb	60 min at 50°C
Random hexamer primers	up to 4 kb	▶ 10 min at 25°C, followed by ▶ 30 min at 55°C
	>4 kb	▶ 10 min at 25°C, followed by ▶ 60 min at 50°C

- 7 Stop the reaction by placing the tube on ice.
- 8 For PCR: Use 1 – 5 µl of the reaction product (first-strand cDNA) as a template for PCR. For initial experiments, try using 2 µl cDNA template for a 50 µl PCR.  
 *If you will not be using the cDNA immediately, you may want to inactivate the RTase and store the cDNA. However, use the guidelines below to determine whether you should do this and how long the cDNA may be stored.*
-  *The cDNA product does not need to be purified before it is used in PCR.*

## 5

### Protocol E: Synthesis of First Strand cDNA with the (Stand-Alone) Transcriptor Reverse Transcriptase

#### About Transcriptor Reverse Transcriptase

Transcriptor Reverse Transcriptase is a fast, new recombinant reverse transcriptase that can complete first-strand cDNA synthesis in just 30 minutes. It is very thermostable (at temperatures as high as 65°C) and can synthesize cDNA as long as 14 kb. Its high thermostability makes it ideal for reverse transcription of GC-rich templates with significant amounts of secondary structure.

 *Transcriptor RTase has an RNase H activity. RNase H removes the RNA template after cDNA synthesis, allowing PCR primers to more easily bind the cDNA and, in some cases, increasing the sensitivity of the PCR (Polumuri et al., 2002).*

#### Additional Equipment and Reagents Required

- ▶ Thermal block cycler with a heated lid
- ▶ Oligo\*, random\* or gene-specific RT primers (see above)
- ▶ PCR Grade Water\*
- ▶ Protector RNase Inhibitor, 40 U/µl\*
- ▶ PCR Nucleotide Mix, 10 mM (each nucleotide)\*

\* available from Roche Applied Science; see Ordering Information (in the Appendix) for details.

## Setting Up and Running the RT Reaction with Transcriptor Reverse Transcriptase



Preheat the thermal block cycler to the temperature of the RT reaction (see step 6 below) before starting the procedure.

### Setting Up the Reaction

- 1
  - ▶ Thaw all frozen reagents before use.
  - ▶ Mix all reagents carefully (by pipetting up and down) and briefly centrifuge them before starting the procedure.
  - ▶ Keep all reagents on ice while setting up the reaction.
- 2 In a sterile, nuclease-free, thin-walled PCR tube (on ice), prepare the template-primer mixture for one 20  $\mu$ l reaction by adding the components in the order listed below. Always wear gloves when handling RNA.

#### Template Primer Mix 1 (for one reaction)

Components	Volume	Final conc.
Total RNA or Poly(A) <sup>+</sup> mRNA	variable	1 $\mu$ g total RNA or 10 ng poly(A) <sup>+</sup> RNA <sup>a</sup>
Primer – choose either		
Oligo(dT) <sub>15</sub> , 100 pmol/ $\mu$ l	0.13 – 1.3 $\mu$ l	13 – 130 pmol/reaction
or Random Hexamers, 0.04 A <sub>260</sub> units/ $\mu$ l	2 $\mu$ l	0.08 A <sub>260</sub> units (3.2 $\mu$ g) / reaction
or Sequence-Specific Primer	variable	0.5 – 2.5 $\mu$ M
PCR Grade Water	variable	to make total volume = 13 $\mu$ l
<b>Total volume</b>	<b>13 <math>\mu</math>l</b>	

<sup>a)</sup> These are the suggested concentrations for initial experiments. Suitable template concentrations may range from 10 ng to 5  $\mu$ g total RNA and from 1 to 100 ng mRNA.

- 3 **Optional Step:** (only needed if template has extensive secondary structure, which will be heat denatured)
  - ▶ Denature the template-primer mixture by heating the tube for 10 min at 65°C in a thermal block cycler with a heated lid (to minimize evaporation).
  - ▶ Immediately cool the tube on ice.
- 4 To the tube containing the template-primer mix, add the rest of the components of the RT mix in the order listed below. (Numbered vials are supplied with the enzyme.)

#### Remainder of RT mix (for one reaction)

Components (Mix 2)	Volume	Final conc.
Transcriptor Reverse Transcriptase Reaction Buffer, 5 $\times$ conc. (vial 2)	4 $\mu$ l	8 mM MgCl <sub>2</sub>
Protector RNase Inhibitor, 40 U/ $\mu$ l	0.5 $\mu$ l	20 U/reaction
PCR Nucleotide Mix, 10 mM each	2 $\mu$ l	1 mM each
Transcriptor Reverse Transcriptase, 20 U/ $\mu$ l (vial 1)	0.5 $\mu$ l	10 U/reaction
<b>Final volume (including template)</b>	<b>20 <math>\mu</math>l</b>	

- 5
  - ▶ Mix the reagents in the tube carefully.
  - ▶ Do not vortex!
  - ▶ Centrifuge the tube briefly to collect the sample on the bottom of the tube.
  - ▶ Place the tube in a thermal block cycler with a heated lid (to minimize evaporation).



- 6 Depending on the primer used and the length of the target mRNA, incubate the RT reaction as described in the table below:

If you are using...	And the target mRNA is..	Incubate the RT reaction...
Anchored oligo(dT) <sub>18</sub> primer or Sequence-specific primer	up to 4 kb	30 min at 55°C
	>4 kb	60 min at 50°C
Random hexamer primers	up to 4 kb	▶ 10 min at 25°C, followed by ▶ 30 min at 55°C
	>4 kb	▶ 10 min at 25°C, followed by ▶ 60 min at 50°C

- 7 Stop the reaction by placing the tube on ice.

- 8 Use 1 – 5 µl of the reaction product (first-strand cDNA) as a template for PCR. For initial experiments, try using 2 µl cDNA template for a 50 µl PCR.

 *If you will not be using the cDNA immediately, you may want to inactivate the RTase and store the cDNA. However, use the guidelines below to determine whether you should do this and how long the cDNA may be stored.*

 *The cDNA product does not need to be purified before it is used in PCR.*

## Protocol F: Synthesis of First Strand cDNA with the Expand Reverse Transcriptase

### About the Expand Reverse Transcriptase

Expand Reverse Transcriptase is a genetically engineered version of the reverse transcriptase from Moloney Murine Leukemia Virus (M-MuLV). The structure of the enzyme and its lack of RNase H activity favor the production of full-length transcripts. Since the RNase H activity of the native enzyme has been reduced below detectable levels (by a point mutation), the engineered enzyme can produce greater amounts of full-length cDNA transcripts than the native M-MuLV enzyme. Expand Reverse Transcriptase can fully transcribe even very long (up to 14 kb) RNA targets.

### Additional Equipment and Reagents Required

- ▶ Thermal block cycler with a heated lid
- ▶ Oligo, random\* or gene-specific RT primers (see above)
- ▶ PCR Grade Water\*
- ▶ PCR Nucleotide Mix, 10 mM (each nucleotide)\*
- ▶ Protector RNase Inhibitor, 40 U/µl\*

\* available from Roche Applied Science; see Ordering Information (in the Appendix) for details.

### Setting Up and Running the RT Reaction with Expand Reverse Transcriptase



Preheat the thermal block cycler to the temperature of the RT reaction (see step 6 below) before starting the procedure.

#### Setting Up the Reaction

- 1
  - ▶ Thaw all frozen reagents before use.
  - ▶ Mix all reagents carefully (by pipetting up and down) and briefly centrifuge them before starting the procedure.
  - ▶ Keep all reagents on ice while setting up the reaction.
- 2 In a sterile, nuclease-free, thin-walled PCR tube (on ice), prepare the template-primer mixture for one 20 µl reaction by adding the components in the order listed below. Always wear gloves when handling RNA. (Numbered vials are supplied with the kit.)

#### Template Primer Mix 1 (for one reaction)

Components	Volume	Final conc.
Total RNA or Poly(A) <sup>+</sup> mRNA	variable	1 µg total RNA or 50 ng poly(A) <sup>+</sup> RNA <sup>a</sup>
Primer – choose either	variable	
Oligo(dT) <sub>n</sub>		20 – 100 pmol
or Random Hexamers		20 – 50 pmol
or Sequence-Specific Primer		10 – 50 pmol
PCR Grade Water	variable	to make total volume = 10.5 µl
<b>Totale volume</b>	<b>10.5 µl</b>	

<sup>a)</sup> These are the suggested concentrations for initial experiments. Suitable template concentrations may range from 10 ng to 5 µg total RNA and from 50 to 100 ng mRNA.

- 3
  - ▶ Denature the template-primer mixture by heating the tube for 10 min at 65°C in a thermal block cycler with a heated lid (to minimize evaporation).
  - ▶ Immediately cool the tube on ice.
- 4 To the tube containing the template-primer mix, add the rest of the components of the RT mix in the order listed below. (Numbered vials are supplied with the enzyme.)

#### Remainder of the RT mix 2 (for one reaction)

Components (Mix 2)	Volume	Final conc.
Expand Reverse Transcriptase Buffer, 5× conc. (vial 2)	4 µl	5 mM MgCl <sub>2</sub>
100 mM DTT (vial 3)	2 µl	10 mM
PCR Nucleotide Mix, 10 mM each	2 µl	1 mM each
Protector RNase Inhibitor, 40 U/µl	0.5 µl	20 U/reaction
Expand Reverse Transcriptase (vial 1)	1 µl	50 U/reaction
<b>Final volume</b> (including template)	<b>20 µl</b>	

- 5
  - ▶ Mix the reagents in the tube carefully.
  - Do not vortex!
  - ▶ Centrifuge the tube briefly to collect the sample on the bottom of the tube.
  - ▶ Place the tube in a thermal block cycler with a heated lid (to minimize evaporation).



- 6 Depending on the primer used, incubate the RT reaction as described in the table below:

If you are using...	Incubate the RT reaction
Oligo(dT) <sub>n</sub>	45 – 60 min at 43°C
Random hexamers	▶ 10 min at 30°C, followed by ▶ 45 min at 42°C
Sequence-specific primer	45 – 60 min at 43°C

- 7 Place the tube on ice.

- 8 Use 1 – 5 µl of the reaction product (first-strand cDNA) as a template for PCR. For initial experiments, try using 2 µl cDNA template for a 50 µl PCR.

 *If you will not be using the cDNA immediately, you may want to inactivate the RTase and store the cDNA. However, use the guidelines below to determine whether you should do this and how long the cDNA may be stored.*

 *The cDNA product does not need to be purified before it is used in PCR.*

### Heat Inactivation and Storage of Reaction Product

If the cDNA product of the reaction will not immediately be used for PCR, store the reaction tube according to the following guidelines:

If The Length of The cDNA is...	Then...	And Store The Tube...
up to 3 kb	Heat inactivate the RTase at 95°C for 2 min	▶ up to 2 h at 2 to 8°C ▶ <b>or</b> >2 h, frozen at -15 to -25°C
>3 kb	Do NOT heat inactivate the RTase	up to 2 h at 2 to 8°C  <i>Do not freeze the tube!</i>

### Troubleshooting the Reverse Transcription

If initial results are unsatisfactory, try the following:

- ▶ Use purified mRNA instead of total RNA as template.
- ▶ Remove potential RT inhibitors by precipitating the template with ethanol, then washing the precipitate with 70% ethanol.
-  *Be sure to remove all traces of ethanol before using the RNA in the RT reaction!*
- ▶ Check the quality of the template on an agarose gel and, if degraded, prepare a new template.
- ▶ Use a sequence-specific primer rather than oligo(dT) or random hexamers.
- ▶ Increase the amount of RNA template in the reaction.
- ▶ Let the RT reaction incubate longer (e.g., if the initial reaction was 30 min, try a 60 min incubation.)

 *For a more detailed list of options, see “Troubleshooting” in the Appendix.*

### 5.4.4 Protocols for the PCR Step

A wide variety of PCR enzymes are available from Roche Applied Science. Each PCR enzyme uses a unique protocol to achieve maximum amplification. Details of these protocols can be found in the articles of Chapter 4. For example, some of the suitable enzymes and protocols, based on length and expected GC content of the cDNA include:

For cDNA of This Length	And This GC Content	Use This Enzyme/Kit	And This Protocol in Chapter 2
up to 3 kb	Normal	Taq DNA Polymerase	Standard PCR
	Normal	FastStart Taq DNA Polymerase	Hot Start PCR
	High	FastStart Taq DNA Polymerase	Hot Start PCR
up to 5 kb	Normal	Expand High Fidelity PCR System	High Fidelity PCR, protocol B
	High	GC-RICH PCR System	Amplification of Difficult Templates
5 to 25 kb	Normal	Expand Long Range dNTPack	Long Template PCR, protocol A
	High	Expand Long Range dNTPack	Long Template PCR, protocol A

 For details on using other PCR enzymes for the second step of RT-PCR, see the PCR Protocol Selection Guide in Chapter 4.

#### Tips on Performing PCR with a cDNA Template

▶ The quality and size of first strand cDNA products can be determined by electrophoresis on a denaturing agarose gel (Sambrook and Russell, 2001).

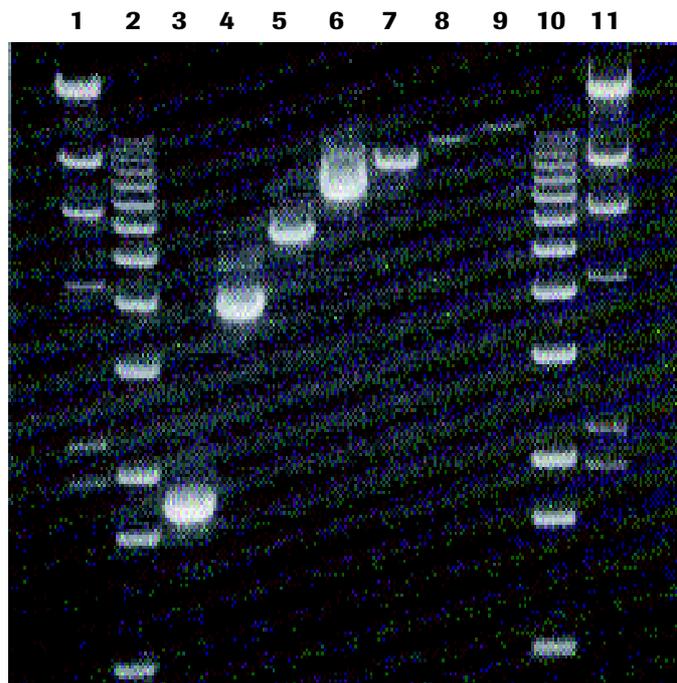
▶ Do not let the volume of the cDNA template exceed 10% of the volume of the PCR mixture (e.g., for a 50 µl PCR mixture, use no more than 5 µl cDNA from the RT reaction). Greater amounts of cDNA may inhibit the PCR.



For initial experiments, try using 2 µl cDNA template for a 50 µl PCR.

▶ When adding Mg<sup>2+</sup> to the PCR mixture, remember that the cDNA product from the RT reaction already contains Mg<sup>2+</sup> ion. For example, if the final concentration of MgCl<sub>2</sub> in the RT reaction is 8 mM (e.g., in Transcriptor Reverse Transcriptase reactions), each µl of the 20 µl cDNA product contributes 8 nmoles of Mg<sup>2+</sup> to the PCR mixture, which translates to 160 µM Mg<sup>2+</sup> in a 50 µl reaction. Adjust the Mg<sup>2+</sup> concentration in the PCR accordingly.

### 5.4.5 Typical Results of Two-Step RT-PCR



**Figure 5.4.1 Two-step RT-PCR amplification of up to 13.5 kb dystrophin targets.** Total RNA (1 µg) from human muscle was reverse transcribed into cDNAs with Expand Reverse Transcriptase, using various primers that were specific for human dystrophin sequences. An aliquot (5 µl) of each unpurified cDNA was amplified with the Expand Long Template PCR System. An aliquot (17 µl) of each PCR product was visualized on an agarose gel. Lanes 1 and 11 contained DNA Molecular Weight Marker II; lanes 2 and 10 contained Molecular Weight Marker X. The lengths of the dystrophin targets were as follows: **Lane 3:** 1857 bp, **Lane 4:** 4041 bp, **Lane 5:** 5893 bp, **Lane 6:** 7678 bp, **Lane 7:** 9556 bp, **Lane 8:** 11.9 kb, **Lane 9:** 13.5 kb

#### Disclaimer

For Titan One Tube RT-PCR System, Cat. Nos. 11 888 382 001, 11 855 476 001; Titan One Tube RT-PCR Kit, Cat. No. 11 939 823 001; Tth DNA Polymerase, Cat. Nos. 11 480 014 001, 11 480 022 001; Protector RNase Inhibitor, Cat. Nos. 11 480 014 001, 11 480 022 001 see Disclaimer No. 1.

For *C.therm.* Polymerase One-Step RT-PCR System, Cat. Nos. 12 016 338 001, 12 016 346 001 see also Disclaimer No. 6.

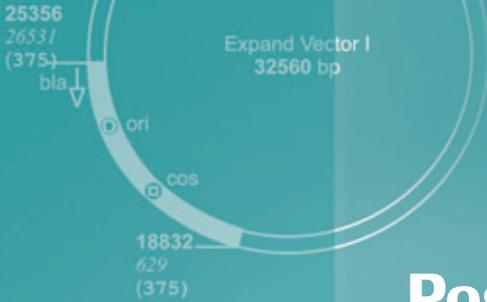
For Pwo DNA Polymerase, Cat. Nos. 11 644 947 001, 22 644 955 001; Pwo SuperYield DNA Polymerase, Cat. Nos. 04 340 868 001, 04 340 850 001, Pwo SuperYield DNA Polymerase, dNTPack, Cat. Nos. 04 743 750 001, 04 743 776 001, and Pwo Master, Cat. No. 03 789 403 001, see Disclaimer No. 2,

For the PCR Core Kit<sup>PLUS</sup>, Cat. No. 11 578 553 001 see Disclaimer No. 4

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# 5

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## Chapter 6

# Post-PCR Purification and Cloning

19399

Expand CLONING SITE  
Linearized, dephos



Expand Vector II  
23494 bp

Expand CLONING SITE  
Linearized, dephos



18832  
629  
(375)

6

<b>6</b>	<b>Post-PCR Purification and Cloning</b>	<b>Page</b>
6.1	Purification of PCR Products.....	159
6.1.1	Purification of PCR Products with the HIGH PURE PCR Product Purification Kit.....	160
6.1.2	HIGH PURE PCR Product Purification Kit: Changed Protocol for Purification of Large DNA Fragments (4.5 to >30 kb).....	163
6.1.3	High Throughput Purification of PCR Products with the HIGH PURE 96 UF Cleanup System.....	167
6.1.4	Elution of PCR Products from Agarose Gel Slices with the Agarose Gel DNA Extraction Kit.....	172
6.2	Cloning of PCR Products.....	175
6.2.1	Overview: Cloning of PCR Products .....	175
6.2.2	Some Useful Kits for Cloning PCR Products.....	178
6.2.3	Procedure for Cloning up to 10 kb PCR Products with the PCR Cloning Kit (Blunt End) .....	183
6.2.4	Procedure for Cloning Long (7– 36 kb) PCR Products with the Expand Cloning Kit .....	184
6.2.5	Activity of Restriction Enzymes in Standard Taq DNA Polymerase Buffer .....	189
6.2.6	Activity of Restriction Enzymes in Pwo SuperYield DNA Polymerase Buffer .....	190



## 6. Post-PCR Purification and Cloning

### 6.1 Purification of PCR Products

After PCR, an amplified product may not be suitable for use in a downstream application (such as cloning or labeling) until it is separated from components of the reaction mix. SuperYieldFailure to remove these components can lead to poor or misleading results.

In such cases, a good nucleic acid purification product can improve your results. Such a product should:

- ▶ Yield a purified product that may be used directly in a downstream application such as sequencing, cloning or labeling.
- ▶ Completely remove proteins and low molecular weight components that can interfere with the downstream procedures.
- ▶ Involve minimal exposure to hazardous chemicals (e.g., phenol).
- ▶ Process a large number of samples quickly and conveniently.

Roche Applied Science offers three products that meet all the above criteria:

- ▶ HIGH PURE PCR Product Purification Kit, for purification of most amplified DNA products from standard reaction mixtures
- ▶ HIGH PURE 96 UF Cleanup System, for high throughput purification of PCR products by ultrafiltration
- ▶ Agarose Gel DNA Extraction Kit, for recovery of PCR products from standard or low melting point agarose gels



*For details on the pack sizes of these products, see the Ordering Information in the Appendix.*

Sections 6.1.1 – 6.1.4 of this chapter explain how to use each of these products to prepare PCR products for downstream procedures.



*Roche Applied Science offers a wide array of products designed to prepare PCR or RT-PCR templates. Of course, these products can also be used to prepare nucleic acids for other common molecular biology procedures. For an overview of other uses for these products plus the latest news about nucleic acids isolation and purification, visit our Nucleic Acid Isolation and Purification (NAPI) special interest site at [www.roche-applied-science/sis/napure](http://www.roche-applied-science/sis/napure).*



### 6.1.1 Purification of PCR Products with the HIGH PURE PCR Product Purification Kit

The principle use for the HIGH PURE PCR Product Purification Kit is the isolation of amplified DNA products as well as cDNA products that are greater than 100 bp (and less than 50 kb) long from a standard reaction mix (up to 100 µl total volume). The protocol described below efficiently removes primers, mineral oil, salts, unincorporated nucleotides and the thermostable DNA polymerase, all of which may inhibit subsequent enzymatic reactions (e.g., labeling, sequencing or cloning of the PCR products).



For details on the available pack sizes of this kit, see the Ordering Information in the Appendix.

#### Overview of Standard Procedure

##### Principle of the Kit

In the presence of chaotropic salt, product DNA binds selectively to glass fiber fleece in a special centrifuge tube. The DNA remains bound while a series of rapid “wash-and-spin” steps remove contaminating small molecules (including small nucleic acids). Finally, low salt elution removes the DNA from the glass fiber fleece. The process does not require DNA precipitation, organic solvent extractions, or extensive handling.

##### Sample Material

Up to 100 µl enzyme reaction solution, containing any of the following:

- ▶ Amplified DNA products that are >100 bp and <50 kb
- ▶ Modified DNA of the same size range from other enzymatic reactions [e.g., DNA processed by restriction enzyme (Lobner *et al.*, 2002), alkaline phosphatase treatment, kinase or other enzymatic reactions (Chang *et al.*, 2001; Salesse *et al.*, 2003)]
- ▶ Hapten-labeled (e.g., DIG-labeled) DNA
- ▶ RNA from *in vitro* transcription reactions
- ▶ First strand cDNA (Footitt *et al.*, 2003)



This application is described in section 3.3.3 of Chapter 3.



With minor modifications, the kit can also be used to isolate DNA from a 100 mg agarose gel slice (D'Errico *et al.*, 2005; Falchetti *et al.*, 2005). For more information on this application, see section 6.1.2 (below) or the package insert for the kit (Cat. No. 11 732 668 001), which is available online at [www.roche-applied-science.com](http://www.roche-applied-science.com).

**Additional Equipment and Reagents Required**

- ▶ Absolute ethanol
- ▶ Standard, tabletop microfuge capable of 13,000 × g centrifugal force
- ▶ 1.5 ml microfuge tubes, sterile

**Time Required**

Total time: approx. 10 min

Hands-on time: less than 10 min

**Purity of Product**

Purified DNA is free of short DNA (<100 bp), small molecules (*e.g.*, primers, salts, unincorporated nucleotides, mineral oil) and proteins (*e.g.*, thermostable DNA polymerase).

**Expected DNA Recovery**

Table 6.1.1 shows the experimentally determined recovery expected from various amounts and sizes of DNA product.

**Table 6.1.1: Expected DNA Recovery from Various Amounts and Sizes of DNA.**

DNA applied (μg)	Recovery (%)	Fragment length (bp)	Recovery (%)	Elution volume (μl)	Recovery (%)
5	77	<100	<5	50	68
10	79	375	>95	100	79
25	80	700	>95	150	80
50	56	3000	>95	200	80



### Standard Procedure for Purification of Products from PCR or Other Enzymatic Reactions

 In the procedure below, all reagents with colored caps are components of the kit. Before starting the procedure, prepare the Wash Buffer solution for use (by adding absolute ethanol) as detailed in the package insert for the kit.

- 
- 1** ▶ Adjust the volume of the DNA sample to 100 µl.  
▶ Add 500 µl Binding Buffer (green cap) to the sample tube.  
▶ Mix the contents of the tube well.
- 
- 2** ▶ Insert one HIGH PURE Filter Tube into one Collection Tube.  
▶ Pipet the entire mixture from Step 1 into the upper buffer reservoir of the Filter Tube.  
▶ Insert the entire HIGH PURE Filter Tube assembly into a standard tabletop microfuge at +15 to +25°C.  
▶ Centrifuge 30 – 60 seconds at maximum speed (approx. 13,000 × g).
- 
- 3** ▶ Remove the Filter Tube from the Collection Tube; discard the flowthrough liquid.  
▶ Again combine the Filter Tube and the used Collection Tube.
- 
- 4** ▶ Add 500 µl Wash Buffer (blue cap) to the upper reservoir of the Filter Tube assembly.  
▶ Centrifuge 1 min at maximum speed (as above).  
▶ Remove the Filter Tube from the Collection Tube; discard the flowthrough liquid.  
▶ Again combine the Filter Tube and the used Collection Tube.
- 
- 5** ▶ Add 200 µl Wash Buffer (blue cap) to the upper reservoir of the Filter Tube assembly.  
▶ Centrifuge 1 min at maximum speed (as above).  
 *This high speed centrifugation step ensures optimal purity and complete removal of any residual Wash Buffer.*
- 
- 6** ▶ Discard the used Collection Tube and any flowthrough liquid it contains.  
▶ Insert the Filter Tube into a clean, sterile 1.5 ml microfuge tube.
- 
- 7** To elute the DNA:  
▶ Add 50 – 100 µl Elution Buffer (colorless cap) to the upper reservoir of the Filter Tube.  
▶ Centrifuge the tube assembly 1 min at maximum speed.  
 *Do not use water for elution, since an alkaline pH is required for optimal yield.*
- 
- 8** The microfuge tube now contains the eluted DNA product, which is suitable for direct use in downstream enzymatic (e.g., cloning, sequencing or labeling) procedures.  
 *If you plan to estimate the DNA yield by determining its  $A_{260}$ , residual glass fibers in the eluate may interfere. To remove these glass fibers, centrifuge the tube containing the eluate at maximum speed for longer than 1 min. Then use only an aliquot of the eluate for the absorbance measurement.*
-

## 6.1.2 HIGH PURE PCR Product Purification Kit: Changed Protocol for Purification of Large DNA Fragments (4.5 to >30 kb)

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### Introduction

The HIGH PURE PCR Product Purification Kit (Roche Applied Science) allows rapid purification of DNA fragments from complex mixtures (including PCRs and restriction nuclease digests) and from agarose gel slices.

Briefly, the kit works as follows. Selective adsorption of the DNA fragments to the fiberglass fleece is achieved in the presence of chaotropes such as guanidine thiocyanate (present in binding buffer 1). Soluble samples such as PCR product mixes are thoroughly mixed with five volumes of binding buffer 1, whereas agarose gel slices are dissolved in three volumes of binding buffer 1 at 55°C [where one volume buffer (in  $\mu\text{l}$ ) equals the weight of the agarose slice (in milligrams)]. According to the standard kit protocol for processing agarose gel slices, 1.5 volumes of isopropanol are added to the samples. The isopropanol-containing solutions are pipetted directly into the upper buffer reservoir of the HIGH PURE Filter Tubes and the tubes are centrifuged for 30 seconds at  $13,000 \times g$ . This binding step is followed by column washing steps (500  $\mu\text{l}$  wash buffer 2) and a final elution step (50  $\mu\text{l}$  or more elution buffer 3).

In this article, a changed protocol is provided that omits the isopropanol-addition step for the purification of large DNA fragments. Furthermore, evidence is provided that in the absence of isopropanol, a higher recovery – in particular of very large DNA fragments (>30 kb) – is obtained, whether DNA is purified from restriction nuclease digest solutions or from agarose gel slices.

### Materials and Methods

The DNA plasmid CMV-pAdEasy-1 (37.2 kb) was isolated from *E. coli* BJ5183-AD-1 cells (AdEasy XL Adenoviral Vector System, Stratagene) that had been grown in Luria Bertani (LB) medium with kanamycin. This low-copy number plasmid was isolated with the Genopure Plasmid Maxi Kit (Roche Applied Science). Restriction-nuclease digest with *Pac* I yields a large 32.7-kb fragment that contains a full-length adenoviral genome along with an expression cassette, and a 4.5-kb fragment that contains a pBR322 origin of replication and a kanamycin-resistance cassette.

Whole restriction nuclease digests or DNA fragments extracted from an agarose gel were separated electrophoretically on 0.8% agarose gels in 1x Tris/acetate/EDTA (TAE) buffer.



## Results and Discussion

To compare DNA material recovered with the standard (+ isopropanol) and the modified (- isopropanol) kit protocols, the plasmid CMV-pAdEasy-1 was digested with *Pac* I and the digest was purified. This restriction digest yields two fragments of 32.7 kb and 4.5 kb, and was chosen for the following reasons:

- ▶ The 32.7 kb fragment is within the upper size limit of DNA fragments usually generated in PCRs or in other applications such as restriction analysis.
- ▶ The larger (32.7 kb) and the smaller (4.5 kb) fragments can be isolated in the same tube, thus allowing a direct comparison of recovery efficiency.

To test whether DNA recovery depended on the amount of material loaded on the HIGH PURE Filter, different amounts of DNA were loaded (3 µg or 1 µg DNA per filter) and analyzed.

Furthermore, since the addition of isopropanol might precipitate large DNA, thereby reducing the binding/ adsorption to the fiberglass, some samples were centrifuged at full speed for 10 minutes after isopropanol was added. The supernatant was loaded onto the filter and processed; any precipitates were directly checked by gel electrophoresis. Other samples were loaded directly onto the filter, without centrifugation, after isopropanol was added.

### Purification from Restriction Nuclease Digest Mix

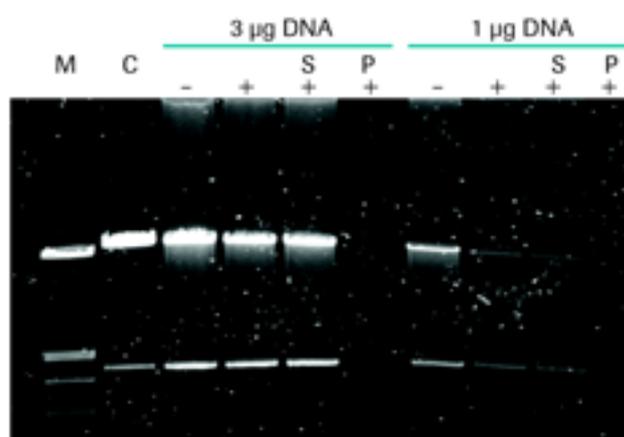
In a total volume of 200 µl, 13.3 µg CMV-pAdEasy-1 DNA was completely digested with *Pac* I. Three 45-µl aliquots (3 µg DNA each) and three 15-µl aliquots (1 µg DNA each) were adjusted with water to 100 µl each. The remaining 20 µl of the digest was used in gel electrophoresis as a control sample.

Next, 500 µl binding buffer 1 was added to each 100 µl sample and the samples were mixed thoroughly. Isopropanol (150 µl) was added to the “+ isopropanol” samples. Samples were centrifuged at  $20,000 \times g$  for 10 minutes, and the supernatants were carefully removed and transferred to new tubes. Pelleted material was dissolved in 50 µl water and analyzed by agarose gel electrophoresis.

After the remaining liquid samples had been applied to HIGH PURE Filter Tubes, the tubes were inserted into collection tubes and the entire HIGH PURE Tube assemblies were centrifuged at  $13,000 \times g$  for 30 seconds. After the flow-through was discarded, 500 µl wash buffer 2 was added to each filter tube and the filter tubes were again centrifuged. Then the filter tubes were removed and inserted into clean 1.5 ml microfuge tubes. To each upper reservoir, 50 µl elution buffer 3 was added and the tube assemblies were centrifuged at  $13,000 \times g$  for 30 seconds. Eluted DNA as well as unpurified control and the isopropanol “pellet” samples were loaded onto an 0.8% agarose gel (containing ethidium bromide) and separated electrophoretically (100 V, 12 hours).

Total DNA recovery can be judged by visual inspection of the intensity of the stained DNA bands in Figure 6.1.1. When the purification protocol was performed without isopropanol, more of the large DNA fragment was recovered. This observation is independent of the amount of DNA loaded on the filter. Interestingly, the addition of isopropanol does not seem to precipitate any DNA material (samples marked “P” in the figure).





**Figure 6.1.1. Recovery of restriction-nuclease-digested plasmid DNA with HIGH PURE PCR Product Purification Kit.** Samples were processed with (+) isopropanol or without (-) isopropanol. Selected samples were centrifuged at full speed after addition of isopropanol (S, supernatant) and the potential precipitates were redissolved in water (P, precipitate). As a control (C), *Pac* I-digested CMV-pAdEasy-1 plasmid DNA was used, yielding 32.7-kb\* and 4.5-kb fragments (M, marker:  $\lambda$  *Hind* III-*Eco* RI DNA).

\* Due to limitations in the resolution of large fragments on 0.8% agarose gels, the 32.7-kb band migrates next to the 21-kb marker of the *lambda Hind* III-*Eco* RI DNA ladder.

### Extraction from Agarose Gel Slices

CMVpAdEasy-1 (13.3  $\mu$ g) was digested with *Pac* I. DNA fragments (32.7 kb and 4.5 kb) from the digest were separated by agarose gel electrophoresis and slices containing each fragment were excised from the gel under UV light illumination.

Three volumes of binding buffer 1 (1,500  $\mu$ l) were added to the slice (500  $\mu$ g) containing the large (32.7 kb) DNA fragment. The agarose slice was completely dissolved at 55°C. Then three 500- $\mu$ l aliquots (“high load” samples) and three 166- $\mu$ l aliquots (“low load” samples) from the dissolved gel slice were processed. Two samples (500  $\mu$ l and 166  $\mu$ l) without isopropanol were loaded directly onto the HIGH PURE Filter; the other samples were mixed with 1.5 volumes isopropanol (187  $\mu$ l and 62.5  $\mu$ l, respectively). Samples were then either loaded onto the HIGH PURE Filter or centrifuged at full speed prior to loading the supernatant. Potential precipitates from this centrifugation were dissolved in water and analyzed directly on the gel.

The remaining steps (washing and elution) were performed according to the standard protocol. Fifty microliters of elution buffer was used.

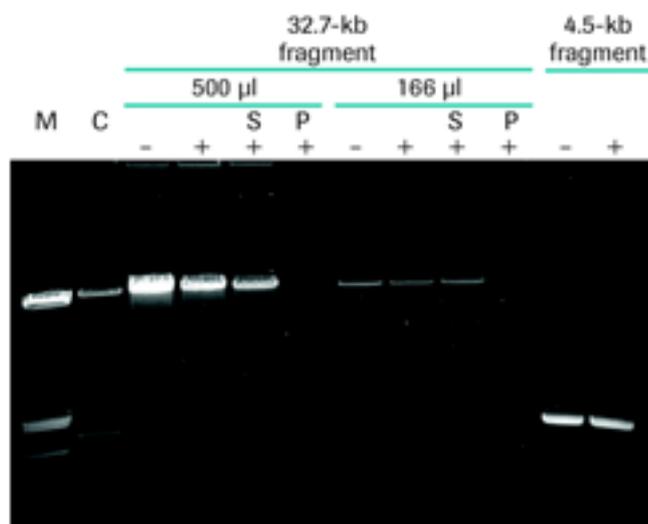
The biological activity of the isolated large DNA fragments, which contained the adenoviral construct, was tested and confirmed by transfecting 293 cells and obtaining infectious viral particles (data not shown).

The smaller (4.5 kb) fragment was obtained in a 312  $\mu$ g slice, therefore 936  $\mu$ l binding buffer 1 was added to it. Then, the agarose slice was dissolved at 55°C. Two 625- $\mu$ l aliquots were removed from the dissolved gel slice and 234  $\mu$ l isopropanol was added to one of the samples. Both samples were loaded directly onto HIGH PURE Filters and processed in parallel with the large 32.7-kb fragment.



More of the 32.7-kb DNA fragment was recovered from HIGH PURE Filters – at least when a larger amount of DNA was loaded – if the sample was not treated with isopropanol (Figure 6.1.2). The difference is not immediately obvious when only small amounts of DNA are loaded. Nevertheless, repeated experiments with low amounts of DNA showed that only the isopropanol-treated samples produced lower recovery rates (data not shown).

There is no visible difference in the recovery of the 4.5 kb fragment with or without isopropanol treatment.



**Figure 6.1.2. Recovery of DNA fragments extracted from agarose gel slices with the HIGH PURE PCR Product Purification Kit.** Samples were processed with (+) isopropanol or without (-) isopropanol. Selected samples were centrifuged at full speed after isopropanol was added (S, supernatant) and the potential precipitates were redissolved in water (P, precipitate). As a control (C), *Pac* I-digested CMV-pAdEasy-1 plasmid DNA was used (M, marker;  $\lambda$  *Hind* III-*Eco* RI DNA).

How can the specific loss of large DNA material (>30 kb) after addition of isopropanol be explained? It seems very likely that the addition of isopropanol alters the hydrophobicity of the binding solution. A more hydrophobic environment, however, does not result directly in precipitation of DNA, as shown by centrifugation of isopropanol-treated samples.

One cannot rule out that isopropanol reduces the adsorption of DNA to the fiberglass, and therefore some material may simply be lost in the flowthrough. This possibility should be checked (e.g., by performing binding studies with radioactively labeled DNA fragments).

## Conclusion

A simple modification of the standard HIGH PURE PCR Product Purification Kit protocols reduces the time required to purify DNA fragments with the kit. When the isopropanol addition step is omitted, the total volume to be passed through the HIGH PURE Filters is reduced. More importantly, when the modified protocol is used, various sizes of DNA fragment are recovered with equal efficiency or – in the case of very large fragments – even greater efficiency than with the standard isopropanol protocol.

### 6.1.3 High Throughput Purification of PCR Products with the HIGH PURE 96 UF Cleanup System

The HIGH PURE 96 UF Cleanup System offers efficient, high throughput purification of PCR products by ultrafiltration. The system can purify up to 96 samples of DNA simultaneously in less than 20 minutes with excellent recovery. The purified products may be used directly in routine procedures (*e.g.*, cloning and labeling) as well as demanding downstream applications such as fluorescent sequencing, detection of single nucleotide polymorphisms (SNPs) and microarray spotting.

The protocols described below remove primers, primer-dimers, salts, unincorporated nucleotides, and other small molecules that may inhibit these downstream procedures.



For details on the available pack sizes of this kit, see the Ordering Information in the Appendix.

#### Overview of System Procedures

##### Principle of the Kit

The HIGH PURE 96 UF Cleanup System uses ultrafiltration and size exclusion to separate the desired DNA fragments from salt and other contaminants (Figure 6.1.3).

In the system, each well of a special 96-well microplate contains a sturdy ultrafiltration membrane, which acts as a molecular sieve. Each PCR product is applied to the ultrafiltration membrane of one well on the plate. Suction or centrifugation is used to draw the liquid from each sample through the membrane. Small molecules (*e.g.*, salts, dNTPs, primers and primer-dimers) pass through the membrane (and into a waste container) readily, while DNA remains on top of the membrane. The DNA can either be washed to ensure removal of small molecules or immediately resuspended in buffer for use in downstream procedures. The procedure does not involve detergents or insoluble reagents that could interfere with, for example, microarray spotting.

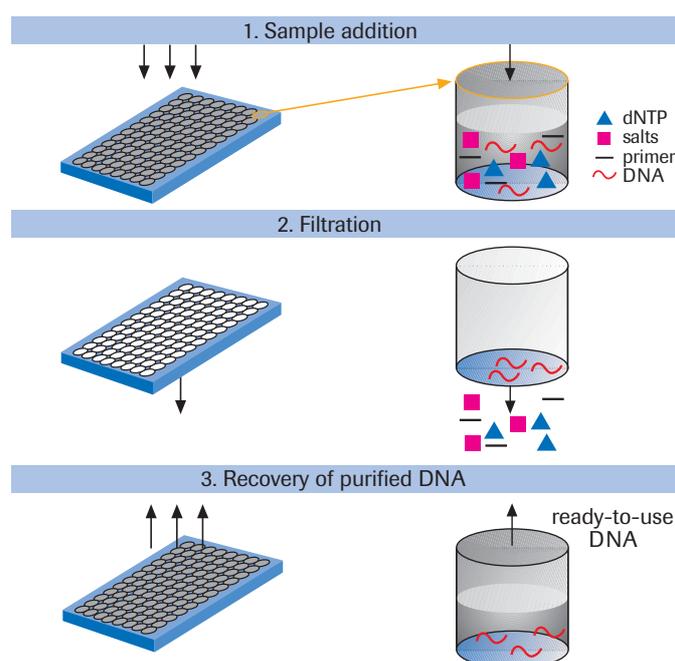


Figure 6.1.3. Schematic: How the HIGH PURE 96 UF Cleanup System purifies PCR products.



### Number of Samples and Sample Size

Each microplate in the system can purify up to 96 samples (amplified DNA products ranging from 100 bp to >10 kb) simultaneously. Unused wells on the plate do not need to be sealed, so the procedure can be run with a partially filled plate.

The volume of each sample can be between 20 and 300 µl. However, the recommended volume for each sample is 100 µl.

### Available System Formats

The HIGH PURE 96 UF Cleanup System is available in two formats:

- ▶ A convenient, “all materials included” kit, which includes Cleanup (ultrafiltration) plates, waste plate, wash and elution buffers, as well as storage plates.



*For a complete listing of the components in the kit, see the package insert for the kit (Cat. No. 04 422 694 001), which is available online at [www.roche-applied-science.com](http://www.roche-applied-science.com).*

- ▶ Individual Cleanup plates without reagents, for the budget-minded or high volume user.

### Additional Equipment Required (If the Complete Kit Is Used)

- ▶ Vacuum manifold capable of sustained vacuum at – 400 to – 600 millibar (mbar)

OR

- ▶ Microplate centrifuge capable of 4,500 × g centrifugal force



*For a list of suitable vacuum manifolds and microplate centrifuges, see the package insert for the kit (Cat. No. 04 422 694 001), which is available online at [www.roche-applied-science.com](http://www.roche-applied-science.com).*

*Since samples are applied to and recovered from the top of the plate, the pipetting procedures can easily be automated using standard liquid-handling machines.*

### Time Required

Manual processing on vacuum manifolds or microplate centrifuges takes approx. 20 min for 96 samples. The time needed for automated processing depends on the instrument used and can be as short as 15 min.



*The indicated times are approximate. The actual time required will depend on the nature and volume of the sample. For example, larger volumes will require longer processing times.*



### Purity and Recovery of Products

Purity of product:  $A_{260\text{nm}}/A_{280\text{nm}} = 1.7 - 1.9$ .

Recovery of product: The amount of DNA recovered depends on the elution volume and the length of the PCR product. For example, the following values were determined by densitometric scanning of the purified products on an agarose gel:

Product size	Recovery rate (%)
150 bp	at least 40
1000 bp	>90
1.5 kb	at least 90
8.0 kb	at least 80

### Quick Reference Procedures for Purification with Vacuum and Centrifugation



*This procedure is adapted from Victor and Walter (2004). For more purification details and tips, see the package insert for the HIGH PURE 96 UF Cleanup Kit, which is available online at [www.roche-applied-science.com](http://www.roche-applied-science.com).*

### Typical Purification Procedure with a Vacuum Manifold

- 1 ▶ Adjust volume of the PCR product mixture to 100  $\mu\text{l}$  with double dist. water (maximum recommended volume for even filtration).
  - ▶ Pipette the mixture directly onto the membrane in one well of a HIGH PURE 96 Cleanup Plate.
- 2 ▶ Place plate atop a suitable vacuum manifold.
  - ▶ Apply vacuum (~400 mbar) until all liquid has passed through the membrane (approx. 10 minutes).
  - ▶ Do either of the following:

**If...**

**Then...**

You want to ensure removal of all small molecules

Go to Step 3 (optional wash).

You want to minimize purification time

Go to Step 4.

- 3 Optional wash:
  - ▶ Vent the manifold for 60 to 90 seconds to release the vacuum.
  - ▶ Dispense 100  $\mu\text{l}$  Wash Buffer (supplied with the kit) into each sample well.
  - ▶ Apply vacuum until all Wash Buffer has passed through (approx. 10 minutes).
  - ▶ Continue applying vacuum for an additional 30 – 60 seconds to dry the membrane.
- 4 ▶ Vent the manifold for 60 to 90 seconds to release the vacuum.
  - ▶ Add 50  $\mu\text{l}$  Resuspension Buffer (supplied with the kit) directly to the center of the membrane in each sample well.
- 5 ▶ Incubate the Resuspension Buffer on the membrane for 5 minutes.
  - ▶ Resuspend the DNA by pipetting the liquid up and down in the well 10 times.
- 6 Use a pipette to remove the resuspended, purified DNA product from each sample well.



### Typical Purification Procedure with a Microplate Centrifuge

- 1 ▶ Adjust the volume of the PCR product mixture to 100  $\mu$ l with double dist. water (maximum recommended volume for an even filtration).  
▶ Pipette the mixture directly onto the membrane in one well of a HIGH PURE 96 Cleanup Plate.
- 2 ▶ Place the plate onto a Waste Collection Plate (supplied with the kit).  
▶ Place the plate “sandwich” in a suitable centrifuge (e.g., the Heraeus Multifuge 3L-R) at 15-25°C.  
▶ Spin the plates for 10 minutes at 4500  $\times$  g.
- 3 ▶ Dispense 100  $\mu$ l Wash Buffer (supplied with the kit) into each sample well.  
▶ Spin the plates for 10 minutes at 4500  $\times$  g.  
 *This wash step is required when you are using a centrifuge. After the first spin, minute amounts of liquid (containing small molecules) will remain atop the membrane, contaminating the sample.*
- 4 ▶ Add 50  $\mu$ l Resuspension Buffer (supplied with the kit) directly to the center of the membrane in each sample well.
- 5 ▶ Incubate the Resuspension Buffer on the membrane for 5 minutes.  
▶ Resuspend the DNA by pipetting the liquid up and down in the well 10 times.
- 6 Use a pipette to remove the resuspended, purified DNA product from each sample well.

### Typical Results

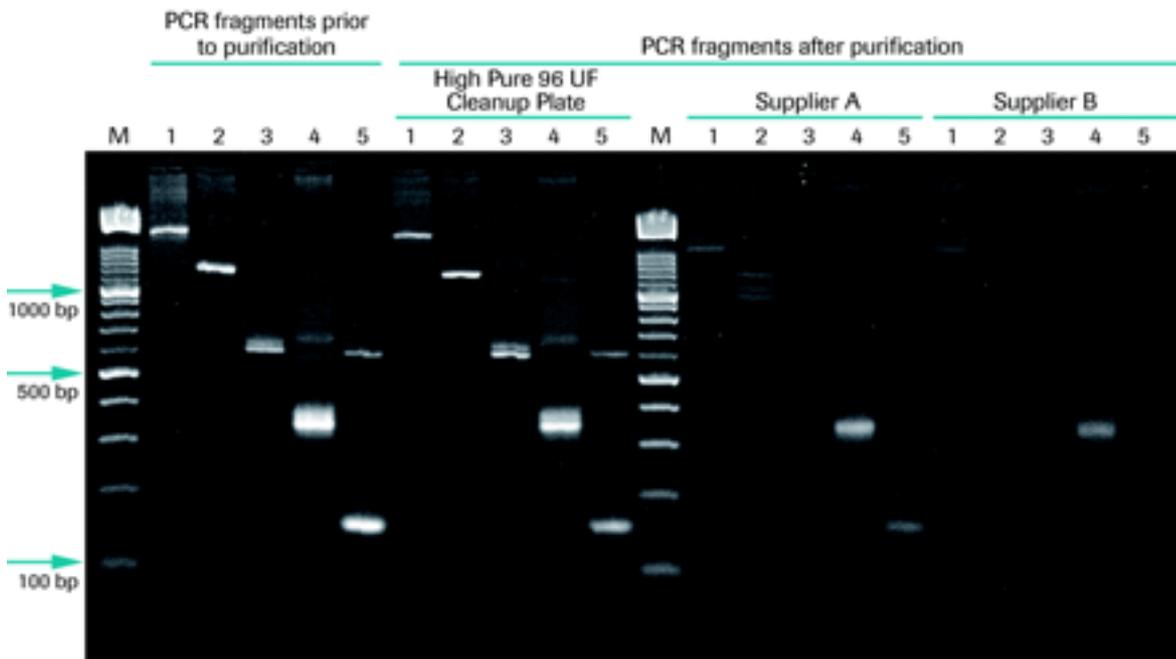
#### Purification with a Vacuum Manifold

PCR products of different lengths were generated. Equal aliquots of the reaction were diluted to a final volume of 100  $\mu$ l and applied to the wells of a HIGH PURE 96 UF Cleanup Plate. Then, the Cleanup Plate was placed atop a suitable vacuum manifold. The DNA samples were purified and recovered as described in the “Typical Purification Procedure with a Vacuum Manifold.”

To evaluate the performance of the kit, two products from different suppliers were also used for purification of the DNA fragments according to the manufacturer’s instructions. Equal amounts of the reaction mix taken before and after purification were analyzed by agarose gel electrophoresis.

As shown in Figure 6.1.4, the HIGH PURE 96 UF Cleanup System may be used to purify a broad range of PCR product sizes (down to 165 bp), with very good recovery results of each fragment. Note that the ultrafiltration kit also efficiently removes contaminating primer-dimers.



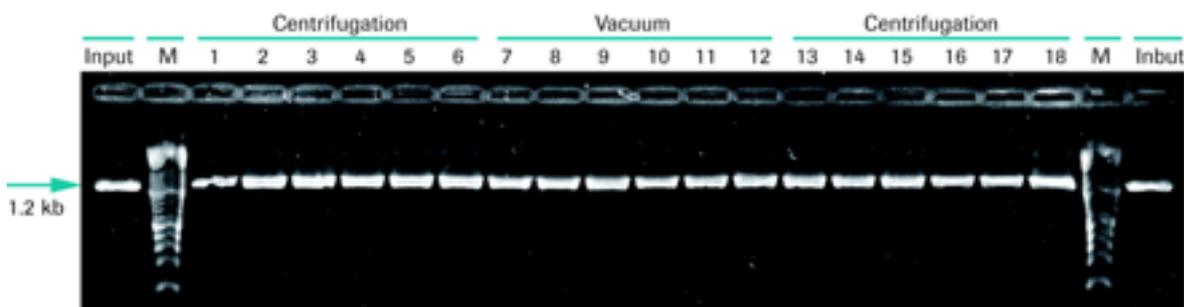


**Figure 6.1.4. PCR products purified with the HIGH PURE 96 UF Cleanup Kit on a vacuum manifold:** Comparison of results obtained with the kit and with products from two other suppliers. Equal amounts of sample were loaded into each well after they were purified with the indicated products. **Lanes 1:** 1.7 kb PCR fragment; **Lanes 2:** 1.2 kb PCR fragment, **Lanes 3:** 600 bp PCR fragment, **Lanes 4:** 350 bp PCR fragment, **Lanes 5:** 165-bp PCR fragment. (M, DNA Molecular Weight Marker XIV). Reprinted from *Biochemica* 3/2004, pages 13-15, July 2004 © Springer-Verlag 2004.

### Purification with a Microplate Centrifuge

Aliquots containing equal amounts of a 1.2 kb PCR fragment were processed with the HIGH PURE 96 UF Cleanup System using a vacuum manifold as described above. Additional aliquots (100  $\mu$ l) were purified with a Heraeus Multifuge 3L-R centrifuge as described in the "Typical Purification Procedure with a Microplate Centrifuge." Equal volumes of solution containing the purified fragments were subjected to agarose gel electrophoresis.

Figure 6.1.5 shows that the HIGH PURE 96 UF Cleanup System gives efficient and robust recovery of PCR fragments, regardless of whether vacuum or centrifugation is used to process the samples.



**Figure 6.1.5. Comparison of ultrafiltration products obtained with vacuum purification and with centrifugation.** Equal amounts of sample were loaded on each well after they were purified with either vacuum (**Lanes 7-12**) or centrifugation (**Lanes 1-6, 13-18**). Samples analyzed in lanes 1-6 and 13-18 were purified on different HIGH PURE 96 UF Cleanup Plates. Unpurified PCR product is shown as Input (M, DNA Molecular Weight Marker XIV). Reprinted from *Biochemica* 3/2004, pages 13-15, July 2004 © Springer-Verlag 2004.

### 6.1.4 Elution of PCR Products from Agarose Gel Slices with the Agarose Gel DNA Extraction Kit

The Agarose Gel DNA Extraction Kit is designed to ensure optimal recovery of DNA fragments such as PCR products or restriction digest fragments from standard or low melting point agarose. The extraction procedure is quick and simple.



*For details on the available pack sizes of this kit, see the Ordering Information in the Appendix.*

#### Overview of Kit Procedure

##### Principle of the Kit

DNA fragments are separated electrophoretically until the DNA fragment of interest is resolved. This fragment is excised in a minimal amount of agarose with a razor blade or scalpel. The agarose is solubilized and the DNA bound to a silica matrix in the presence of a chaotropic salt. Since the binding process is specific for nucleic acids (Vogelstein and Gillespie, 1979), the bound molecules can be separated and purified from impurities with simple washing steps. The DNA is then eluted from the matrix in low salt buffer or water. The recovered product is free of small particles and is ready-to-use in downstream applications.



*For a complete listing of all reagents in the kit, see the package insert for the kit (Cat. No. 11 696 505 001), which is available online at [www.roche-applied-science.com](http://www.roche-applied-science.com).*

##### Sample Size and Type

The standard protocol extracts DNA from a 100 mg agarose gel slice.

For optimal recovery, the extracted DNA fragments should be between 0.4 and 9.5 kb. However, if lower recovery rates are acceptable, the uniformity of the spherical particles in the silica matrix allows isolation of large, intact DNA fragments up to 100 kb in length. The narrow size distribution of the silica particles and the absence of fine material also allow small DNA fragments to be bound tightly to the matrix. Thus even oligonucleotides (>20 nucleotides) can be recovered from agarose gels, free of fine particles that might inhibit subsequent enzymatic reactions.



### Additional Equipment and Reagents Required

- ▶ Standard equipment for agarose gel electrophoresis
- ▶ Standard or low-melting point agarose (e.g., Agarose MP\*, Agarose LE\*, Agarose MS\*)



*The kit will work with different agaroses; you do not need low melting point agarose.*

- ▶ TAE or TBE gel electrophoresis buffer
- ▶ Sharp scalpel or razor blade
- ▶ 1.5 ml microfuge tubes
- ▶ Tabletop centrifuge
- ▶ TE buffer (10 mM Tris-HCl; 0.1 mM EDTA, pH 8.0 – 8.5) or double-distilled water

\* available from Roche Applied Science; see Ordering Information (in the Appendix) for details.

### Time Required

Extraction requires approx. 45 min and involves few hands-on steps.

### Purity and Recovery of Products

Recovered fragments are readily cut by restriction enzymes; no inhibition of digestion is seen. Isolated DNA fragments may be efficiently ligated into cloning vectors or labeled to high specific activity via random primed labeling or nick translation.

Typical recovery rates for smaller and larger DNA fragments are:

Product size	Recovery rate (%)
20 bp	55
40 bp	68
120 bp	76
200 bp	80
8 – 9 kb	75
>10 kb	<60



**Standard Procedure for Extraction of PCR Product from an Agarose Gel Slice**

 Add absolute ethanol to the Washing Buffer before using it. For details, as well as tips on extraction, see the package insert for the Agarose Gel DNA Extraction Kit (Cat. No. 11 696 505 001), which is available online at [www.roche-applied-science.com](http://www.roche-applied-science.com).

- 1  Electrophorese the PCR product mixture on an agarose gel in TAE or TBE buffer.
  -  With a sharp scalpel or razor blade, excise the interesting DNA band from the gel, taking only the minimal amount of agarose necessary to recover the band.
  -  Weigh the excised gel slice in a preweighed 1.5 ml microfuge tube.

---

- 2  For each 100 mg of agarose gel in the slice, add 300 µl Agarose Solubilization Buffer (vial 2 from the kit) to the tube.
  -  Double the amount of buffer used if the gel is >2% agarose.

---

- 3  Resuspend the Silica Suspension (vial 1) until it is homogeneous.
  -  Add 10 µl of the homogeneous silica suspension to the tube containing the slice.

---

- 4  Incubate the mixture for 10 min at 56 - 60°C, vortexing it briefly every 2 - 3 min.

---

- 5  Centrifuge the tube in a tabletop centrifuge at maximum speed for 30 seconds.
  -  Discard the supernatant.

---

- 6  Use a vortex mixer to resuspend the silica suspension (which contains the DNA) in 500 µl Nucleic Acid Binding Buffer (vial 3).
  -  Centrifuge the tube and discard the supernatant as in Step 5.

---

- 7  Wash the pellet with 500 µl working Washing Buffer solution (vial 4).
  -  Centrifuge the tube and discard the suspension as in Step 5.

---

- 8 Repeat Step 7.

---

- 9  Remove all the liquid with a pipette.
  -  Invert the tube on an adsorbent tissue and allow the pellet to dry at room temperature for 15 minutes.

---

- 10 To elute the DNA:
  -  Add 20 - 50 µl of TE buffer (pH 8.0 - 8.5) or double distilled water (pH 8 - 8.5) to the dry pellet.
  -  Resuspend the pellet by vortexing.
  -  Incubate the suspension for 10 minutes at 15 to 25°C (or 15 - 20 minutes at 56 to 60°C, for >5 kb fragments), vortexing the tube briefly every 2 -3 minutes.
  -  Centrifuge the tube at maximum speed for 30 seconds.
  -  Transfer the supernatant (which now contains the extracted DNA) to a new tube.
  -  Be careful not to transfer any of the silica matrix with the supernatant.

## 6.2 Cloning of PCR Products

To ensure maximum efficiency in various downstream applications (*e.g.*, gene expression, *in vitro* transcription, sequencing, preparation of labeled hybridization probes) PCR products are often cloned into appropriate vectors. This chapter presents both a brief review of cloning strategies, plus product information and procedures for simple, high efficiency cloning of PCR products.

### 6.2.1 Overview: Cloning of PCR Products

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Cloning of amplified DNA is often a difficult step in analyzing the products of a polymerase chain reaction. To facilitate cloning of PCR products, several techniques have been developed.

To choose a method, consider several factors, *e.g.* the purpose of the cloning experiment, the type of DNA polymerase used, and the length of the PCR product. If, for example, a DNA polymerase with proofreading activity (*e.g.*, Pwo DNA Polymerase) is used for a site-directed mutagenesis study, blunt-end cloning may be suitable. On the other hand, for protein expression, a method that allows directional cloning is useful.

This article gives a brief overview of some of the cloning strategies that have been developed for cloning PCR products.

#### TA Cloning

Taq DNA Polymerase has a terminal transferase activity that preferentially adds adenine to the 3' ends of PCR products (Clark, 1988; Hu, 1993). PCR products with such a single 3' adenylate extension can be cloned into a vector containing complementary 3' thymidine overhangs (TA cloning). TA cloning may also be used for the products of Tth DNA Polymerase and the Expand PCR Systems.

The so-called T-vectors can be made by using one of the restriction enzymes (*e.g.*, *Xcm* I) that generate a 3' end with a single base protruding (Mead *et al.*, 1991; Kwak and Kim, 1995; Harrison *et al.*, 1994; Borovkov and Rivkin, 1997). However, because *Xcm* I has a rare recognition sequence, the *Xcm* I restriction site has to be introduced by insertion of a synthetic oligonucleotide into the multiple cloning site.

Alternatively, T-vectors can be prepared by enzymatic addition of a single thymidylate residue with either terminal transferase and ddTTP (Holton and Graham, 1991) or Taq DNA Polymerase and dTTP (Marchuk *et al.*, 1991). Polymerases used for the TA cloning method must have terminal transferase activity and lack 3' – 5' exonuclease activity. Besides Taq DNA Polymerase, several DNA polymerases (*e.g.*, Tth DNA Polymerase) have these characteristics and can therefore be used for TA cloning.



## Blunt-End Cloning

DNA polymerases (e.g., Pwo SuperYield DNA Polymerase) with 3' – 5' exonuclease (proofreading) activity remove mismatched nucleotides from 3' ends of double-stranded DNA and generate blunt-ended PCR products (Hu, 1993). The PCR products generated by these proofreading polymerases can be cloned into vectors by blunt-end ligation (Lohff and Cease, 1992).

Polishing protocols allow removal of a single nucleotide extension from PCR products generated with Taq DNA Polymerase; these polished PCR products may also be cloned by blunt-end ligation into a suitable vector (Lohff and Cease, 1992; Costa and Weiner, 1994).

Generally, however, blunt-end cloning of PCR products (or any DNA) into plasmid vectors is less efficient than sticky-end cloning. Therefore a more efficient variation of the blunt-end cloning method has been developed. In this method, a rare cutter restriction enzyme is added to the ligation mixture to linearize any self-ligated vector formed during the ligation reaction (Costa *et al.*, 1994).



*To improve the efficiency of blunt-end cloning, use positive selection systems like the PCR Cloning Kit (blunt end) from Roche Applied Science, which is described in section 6.2.2 below.*

## Directional Cloning through Modified Primers

Several cloning methods require modification of PCR primers to improve cloning efficiency and to facilitate directional cloning of the PCR products.

One common method for efficient directional cloning is to introduce additional restriction sites at the 5' end of each of the primers. As amplification proceeds, these primers are incorporated into the PCR product. After PCR, the amplified DNA fragment is digested with the appropriate restriction enzyme and ligated into the multiple cloning site of a linearized vector (Scharf *et al.*, 1986).

However, there are two potential problems with this approach. Some restriction enzymes fail to cleave at sequences located near the ends of linear double-stranded DNA (Kaufman and Evans, 1990; Jung *et al.*, 1990). Furthermore, since the sequence of the PCR product is often unknown, the chosen restriction enzyme could potentially recognize and cleave sites within the product itself.

Another method which uses modified primers is ligation-independent cloning (Aslanidis and de Jong, 1990; Shuldiner *et al.*, 1991).

In Uracil-DNA-Glycosylase-mediated cloning, the 5' ends of the PCR primers are partially complementary to vector sequences and contain several dUMP residues. Incorporation of these primers during PCR results in selective placement of dUMP residues at the 5' end of amplification products. After the dUMP residues are removed with Uracil-DNA Glycosylase, resulting apyrimidinic sites are susceptible to cleavage by heat and alkaline conditions. The resulting single-stranded 3' ends can then be annealed to complementary ends of the vector and the resulting chimeric DNA molecules transformed without *in vitro* ligation (Rashtchian *et al.*, 1992).

One example of directional cloning which does not require special primers is Exonuclease-III-mediated directional cloning. Under controlled reaction conditions, the 3' – 5' exonuclease activity of Exonuclease III can degrade the PCR product from the 3' ends of both strands. The resulting modified PCR product can easily be cloned into a linearized vector with complementary bases (Kaluz *et al.*, 1992; Walls *et al.*, 1993).

## Cloning Long DNA

By using blends of different DNA polymerases (Barnes, 1994; Cheng, 1994), researchers have been able to amplify long DNA targets. However, it is difficult to use plasmid-based vectors for cloning such large (greater than 10 kb) PCR products because the cloning capacity of vectors is limited.

Efficient cloning of long DNA fragments can only be achieved with cosmid vectors and packaging systems. For example, a cosmid-based method has been developed that allows cloning of up to 36 kb PCR fragments with high efficiency.



*For an example of a cosmid-based procedure, see section 6.2.4 of this chapter.*

## Positive Selection Vectors

Very often blue/white screening is used to identify transformed bacteria that contain recombinant plasmids. This method allows non-recombinants and recombinants to be distinguished by colony color. However, in-frame cloning of the PCR fragment into the X-gal gene sometimes allows the cloned gene to be expressed, leading to leaky phenotypes and variation in the intensity of blue colonies. This phenomenon leads to the generation of false negative colonies.

In order to obtain maximal transformation efficiency, several positive selection vectors have been developed. For most positive selection vectors, the selection principle is based on lethal genes (Bernard *et al.*, 1994; Henrich and Schmidtberger, 1995; Yazynin *et al.*, 1996; Schlieper *et al.*, 1998). However, genes that convey sensitivity to toxic reagents have also been described (Kast, 1994). The insertion of foreign DNA disrupts expression of the lethal gene, so only those clones that contain an insert survive.

The use of such positive selection vectors is limited by their restricted host range, and by the need for mutagenic agents or complex media (Kast, 1994). However, Schlieper *et al.* (1998) have developed a positive selection vector that can be used with almost all commonly used strains of *E. coli* without requiring complex media or induction conditions. In this method, blunt-end DNA fragments (generated by PCR or other methods) are ligated into the *Mlu* NI restriction site within the multiple cloning site of the vector, disrupting expression of the *crp<sup>S</sup>* gene. Only positive recombinants grow after transformation, since cells that harbor the non-recombinant lethal gene are killed during the transformation.



*For an example of a procedure which uses this positive selection vector, see section 6.2.3 of this chapter.*



## 6.2.2 Some Useful Kits for Cloning PCR Products

This section presents an overview of three Roche Applied Science kits that simplify high efficiency cloning of PCR products. These kits are:

- ▶ PCR Cloning Kit (blunt end)
- ▶ Expand Cloning Kit
- ▶ Rapid DNA Ligation Kit



For details on available pack sizes for these kits, see the Ordering Information in the Appendix.

### PCR Cloning Kit (Blunt End)

The PCR Cloning Kit (blunt end) uses a positive selection method that enhances recovery of recombinant clones (since clones that do not contain an insert will not grow). The kit may be used to:

- ▶ Clone blunt-end DNA fragments (e.g., PCR products generated with Pwo SuperYield DNA Polymerase) that are up to 10 kb long.
- ▶ Clone PCR products with one-base overhangs [e.g., those generated with Taq or Tth DNA Polymerase (Hu, 1993; Clark, 1988) or the Expand High Fidelity PCR System] if they are first polished with T4 DNA Polymerase (to form blunt ends).
- ▶ Add T7 and SP6 promoter sites (using primers supplied in the kit during PCR) for subsequent *in vitro* transcription of cloned inserts.



These primers also allow bacterial clones produced with the kit to be directly amplified by PCR (without purification of the plasmid DNA) to screen for the desired insert (Güssow and Clackson, 1989).

### Principle of the Kit

The kit contains a specially designed high copy number positive selection vector (pCAP<sup>S</sup>) which contains the lethal mutant gene *crp<sup>S</sup>* for an altered catabolite gene activator protein (CAP<sup>S</sup>) (Schlieper *et al.*, 1998). Ligation of the blunt-ended DNA fragment, generated by PCR or other methods, into the *Mlu* NI restriction site disrupts the expression of the gene. Only positive recombinants grow after transformation, since cells that harbor the non-recombinant lethal gene are killed during the transformation event. PCR primers (included in the kit) that contain promoter sites for T7 and SP6 RNA Polymerase allow the cloned fragments to be amplified for subsequent *in vitro* transcription (Sambrook and Russell, 2001).

The kit will work with a broad range of competent *E. coli* strains, because the plasmid may be transformed into any competent *E. coli* strain that has an active adenylate cyclase gene (*cya*<sup>+</sup>), e.g. DH5-alpha or any JM10 strain. [The *crp<sup>S</sup>* - induced lethality is not observed in *E. coli* strains that have a defective adenylate cyclase gene (genotype *cya*<sup>-</sup>) and thus are unable to produce the coactivator, cyclic AMP. The observed lethality seems to be connected to the DNA binding activity of the CAP<sup>S</sup> x cAMP complex (Lupata *et al.*, 1997.)



For a complete list of the components in this kit, see the package insert for the PCR Cloning Kit (blunt end) (Cat. No. 11 939 645 001), which is available online at [www.roche-applied-science.com](http://www.roche-applied-science.com). For a detailed procedure that uses this kit, see section 6.2.3.

### Key Information about the Kit

Starting sample: Any blunt-ended DNA, up to 10 kb.

Time required for the complete procedure: 2.0 – 2.5 h (+ overnight growth of transformed cells).



*The ligation reaction requires only 5 minutes and the transformation only 90 minutes.*

### Cloning PCR Products with the Kit

Figure 6.2.1 summarizes the blunt end cloning procedures possible with the kit. Figure 6.2.2 shows the structure of the pCAP<sup>s</sup> plasmid supplied in the kit.

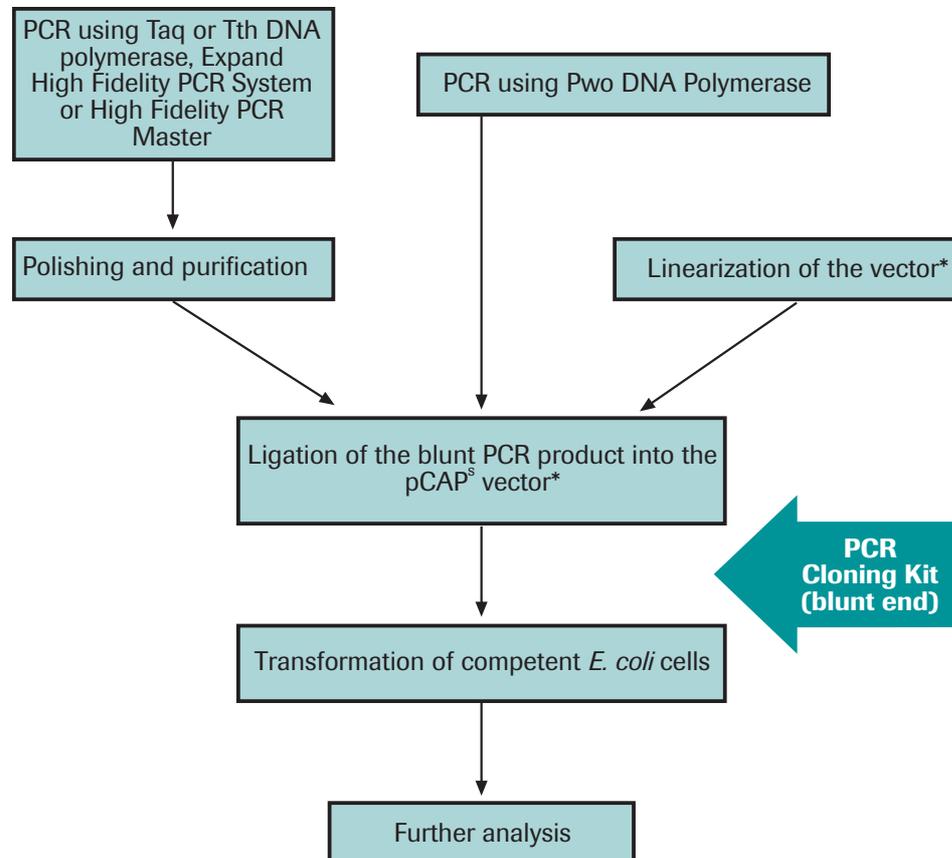
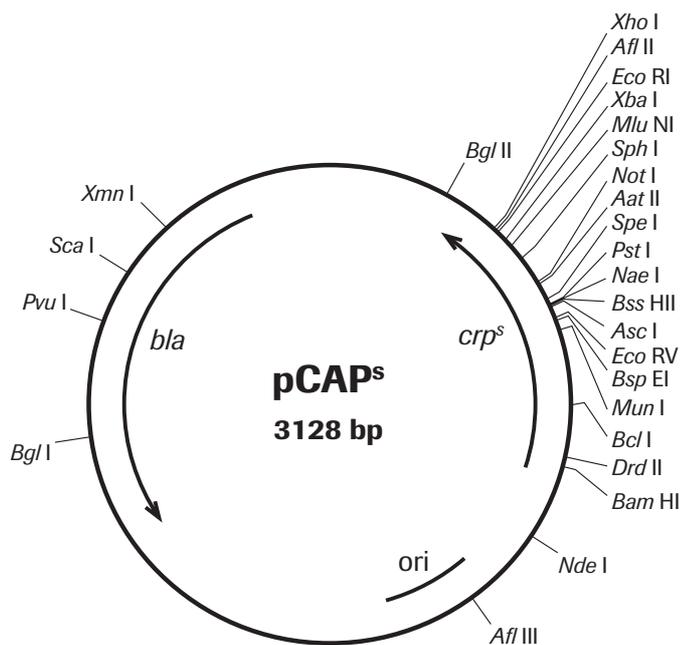


Figure 6.2.1. Flow chart for blunt-end cloning with the PCR Cloning Kit (blunt end). \* = stopping points.



**Figure 6.2.2. Structure of pCAP<sup>S</sup> plasmid (3128 bp).** ori: origin of replication; *bla*:  $\beta$ -lactamase gene (ampicillin resistance); *crp<sup>S</sup>*: gene coding for the cyclic AMP receptor protein mutant. All restriction sites shown are unique. The EMBL Nucleotide Database Accession Number of pCAP<sup>S</sup> is AJ001614.

### Expand Cloning Kit

The Expand Cloning Kit uses cosmids and a lambda packaging system (Hohn, 1980) to efficiently clone large (7–36 kb) DNA fragments (e.g., PCR products generated with the Expand PCR Systems) (Barnes, 1994; Cheng, 1994). The fragments may have either blunt ends or ends with 5'- or 3'-overhangs. The lambda packaging system and the Expand vector limit the size of fragment that can be cloned, thereby preventing the cloning of undesirable concatemers of the PCR products (Hohn, 1975; Hohn, 1977).

The cosmid vectors in the kit also contain several features that simplify analysis of the cloned insert. The cloned Expand insert can easily be excised from the vectors by using the restriction sites for any of four “rare cutter” restriction enzymes contained in the vector. For *in vitro* transcription, the Expand vectors contain the promoters for two RNA Polymerases (Brown, 1986), so the cloned product can easily be transcribed *in vitro*. The vectors also contain M13 primer sites, which simplify the sequencing of the cloned insert.

### Principle of the Kit

The kit uses specially designed cosmid vectors (Expand vectors I, II, and III) of different sizes. A polishing reaction (Wang, 1994) of the PCR fragments must be performed before cloning in order to remove the non-template extra nucleotide residues at the 3' end, which are added by Taq DNA Polymerase. Either the polished PCR fragment or the blunt-end PCR fragments (7–36 kb) are inserted into an Expand vector by blunt-end cloning. The recombinant construct is then packaged into lambda bacteriophages with DNA packaging extracts. These lambda phage heads can infect an *E. coli* magnesium culture. Since the gene for ampicillin resistance is present on the Expand vectors, positive clones can be selected on agar plates that contain ampicillin.



For a complete list of the components in this kit, see the package insert for the Expand Cloning Kit (Cat. No. 11 940 392 001), which is available online at [www.roche-applied-science.com](http://www.roche-applied-science.com). For a detailed procedure that uses this kit, see section 6.2.4.

### Key Information about the Kit

Starting sample: PCR product or other DNA fragment, 7 – 36 kb long.

Time required for the complete procedure: approx. 9 h (+ overnight growth of cells).

#### Cosmid characteristics:

- ▶ Expand vector I (32.6 kb) for cloning 7.0 – 16.5 kb DNA fragments
- ▶ Expand vector II (23.5 kb) for cloning 16.5 – 25.0 kb DNA fragments
- ▶ Expand vector III (15.2 kb) for cloning 25.0 – 36.0 kb DNA fragments

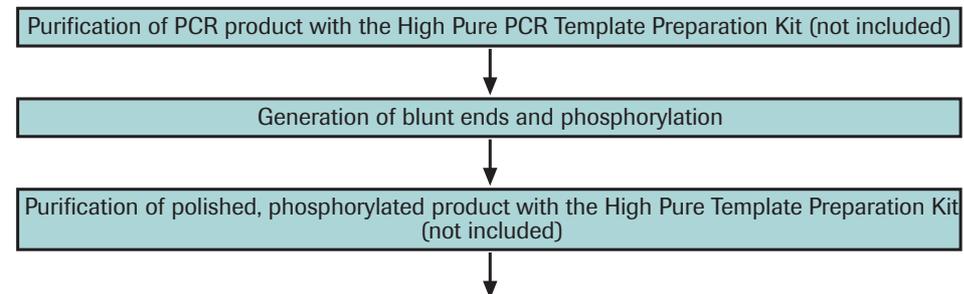


All vectors contain the gene for ampicillin resistance, SP6 and T7 promoter sites (for *in vitro* transcription), M13 universal primer sites (for sequencing), and sites for four “rare cutter” restriction enzymes (*Not* I, *Swa* I, *Sfi* I, and *Spe* I) (for easy removal of cloned DNA from vector).

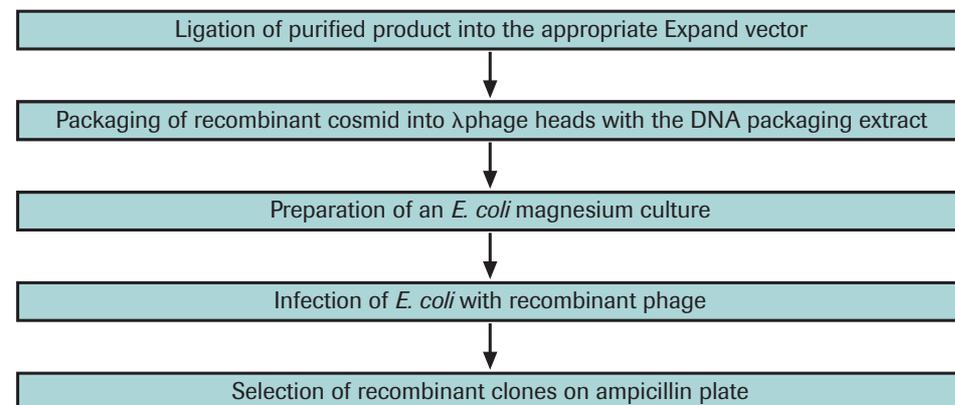
### Cloning PCR Products with the Kit

Figure 6.2.3 summarizes the cloning procedures possible with the kit. Figures 6.2.4 and 6.2.5 show structural details of the Expand cosmid vectors.

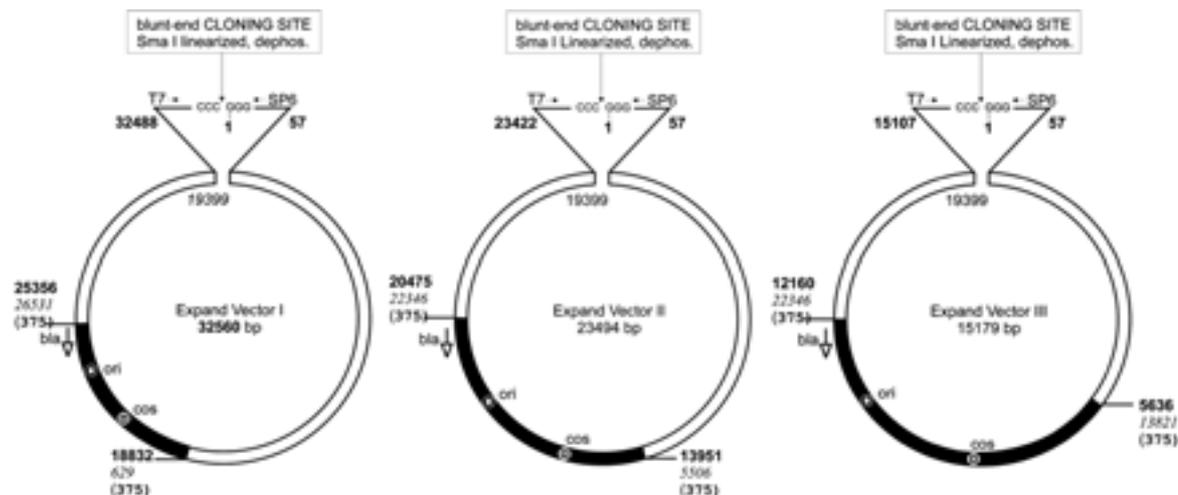
#### Preparation of the Expand PCR DNA Fragments



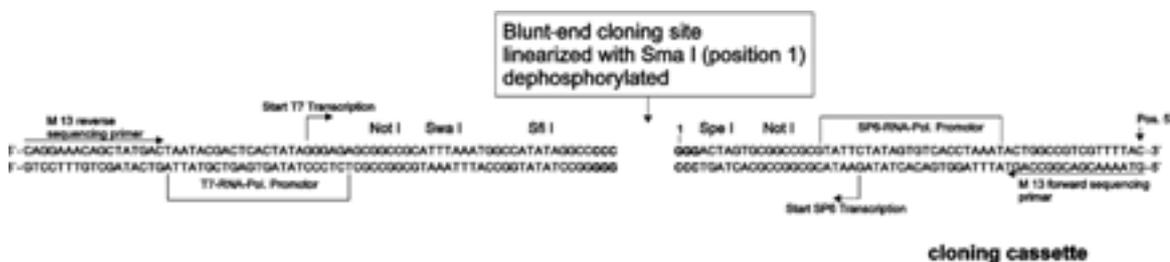
#### Cloning of the prepared Expand PCR Fragments



**Figure 6.2.3.** Flow chart for cloning with the Expand Cloning Kit. For details of the cloning procedure, see section 6.2.4 in this chapter.



**Figure 6.2.4. Structure of Expand cosmid cloning vectors.** The filled part of each vector is derived from pHC79. The open part of each vector is derived from the lambda phage DNA, which is replaced after the DNA fragment is successfully cloned. Nucleotide positions shown in **bold face** type (e.g., **25356**) are from the Expand vector. Positions shown in *italic* type (e.g., *26531*) are from the lambda phage DNA. Positions shown in parentheses [e.g., (375)] are from pHC79.



**Figure 6.2.5. Structure and sequence of blunt-end cloning sites in all Expand cosmid cloning vectors.**

### Rapid DNA Ligation Kit

The T4 DNA ligase in the kit can ligate either sticky-end or blunt-end DNA fragments into a cloning vector. The buffers in the kit allow ligation to be completed in just 5 minutes. Reaction conditions can be adjusted to produce either circular or concatemeric products. The kit may be used for:

- ▶ Cloning PCR products into plasmid or phage vectors.
- ▶ Addition of linkers.
- ▶ Circularization of linear DNA.



*Circularized DNA may be introduced into E. coli cells by electroporation.*

### Key Information about the Kit

Components of the kit: The kit contains ligase and buffers. For a complete list of kit components, see the package insert for the Rapid DNA Ligation Kit (Cat. No. 11 635 379 001), which is available online at [www.roche-applied-science.com](http://www.roche-applied-science.com).

Starting sample: PCR product or other DNA fragment with either blunt or sticky ends.

Time required for the ligation: 5 minutes.

Incubation temperature: 15 to 25°C.

### Procedures

For step-by-step ligation, bacterial transformation and electroporation procedures, see the package insert for the kit (Cat. No. 11 635 379 001), which is available online at [www.roche-applied-science.com](http://www.roche-applied-science.com).

### 6.2.3 Procedure for Cloning up to 10 kb PCR Products with the PCR Cloning Kit (Blunt End)

This procedure describes the cloning of both blunt-end and sticky-end PCR products with components from the PCR Cloning Kit (blunt end).



*For details of this procedure as well as a procedure for amplifying the cloned DNA, see the package insert for the PCR Cloning Kit (blunt end) (Cat. No. 11 939 645 001), which is available online at [www.roche-applied-science.com](http://www.roche-applied-science.com).*

- 1 Is the PCR product to be cloned blunt-ended or sticky-ended (containing single-stranded overhangs at the ends)?

It is	Then...
blunt-end DNA (e.g., produced with Pwo SuperYield DNA Polymerase or cut with an enzyme that generates blunt ends)	It is ready to be cloned. Go to Step 3.
sticky-end DNA (e.g. produced with Taq or Tth DNA Polymerase or the Expand High Fidelity PCR System or cut with a restriction enzyme that generates sticky ends)	The ends must be polished before cloning. Go to Step 2.



- 2** To polish the ends of a DNA with single stranded overhangs (sticky ends), do the following:
- ▶ To a sterile 1.5 ml reaction tube, add the following components in the order listed:

Component	Volume
PCR product, sticky-end <sup>a</sup>	Variable
T4 DNA Polymerase Reaction Buffer, 5× conc.	4 μl
PCR Grade Nucleotide Mix, diluted to 0.1 μM each	2 μl
T4 DNA Polymerase, 1 unit/μl	1 μl
Sterile, double distilled water	Variable, to make final volume = 20 μl
<b>Total volume</b>	<b>20 μl</b>

<sup>a)</sup> Use enough DNA to ensure the amount of product obtained will be sufficient for the ligation step. (See Step 4 below.)

- ▶ Incubate the tube for 30 min at 37°C.
- ▶ Purify the polished DNA with the HIGH PURE PCR Product Purification Kit.
- ▶ Use the purified, blunt-end DNA in Step 4.

- 3** Linearize the pCAP cloning vector as follows:
- ▶ To a sterile 1.5 ml reaction tube, add the following components in the order listed:

Component	Vial (from the Kit)	Volume
pCAP vector, 5 ng/μl	1	2 μl
SuRE/Cut Buffer A, 10× conc.	3	1 μl
Restriction Endonuclease <i>Mlu</i> NI, 1 unit/μl	2	1 μl
Sterile, double distilled water		6 μl
<b>Total volume</b>		<b>10 μl</b>

- ▶ Incubate the tube for 5 min at 37°C.
- ▶ Inactivate the enzyme by heating the tube for 15 min at 65°C
- ▶ Chill the tube on ice briefly, until it reaches room temperature.

- 4** To ligate the blunt-end PCR product (from Step 1 or Step 2) to the linearized vector (from Step 3), do the following:

- ▶ To a sterile 1.5 ml reaction tube, add the following components in the order listed:

Component	Vial (from the Kit)	Volume
Linearized pCAP Vector, 1 ng/μl (from Step 3) <sup>a</sup>		1 μl
T4 DNA Dilution Buffer	6	2 μl
T4 DNA Ligation Buffer	5	10 μl
T4 DNA Ligase, 5 units/μl	7	1 μl
Blunt-end PCR product (from Step 1 or Step 2) <sup>a,b</sup>		1 – 6 μl
Double distilled water		Variable, to make final volume = 20 μl
<b>Total volume</b>		<b>20 μl</b>

<sup>a)</sup> The molar ratio of vector to blunt-end DNA should be between 1: 100 and 1: 500 in the ligation reaction.

<sup>b)</sup> To determine the amount of PCR product needed, use the following formula:

$$\left[ \frac{\text{ng of vector} \times \text{kb size of insert}}{\text{kb size of vector}} \right] \times \left[ \text{molar ratio of } \frac{\text{insert}}{\text{vector}} \right] = \text{ng of insert}$$



- 
- ▶ Incubate the tube for:
    - ▶ 5 min at 15 to 25°C, if the PCR product is 5 kb or less
    - ▶ Up to 30 min at 15 to 25°C, if the PCR product is >5 kb
  - ▶ Centrifuge the tube briefly.
  - ▶ Place the ligated DNA on ice until ready to perform the transformation step (Step 7).
    - ! *The ligated DNA may also be stored at -15 to -25°C.*
- 

- 5** For each transformation to be performed, prewarm 360 µl of SOC medium to 37°C. (SOC media is: 0.5% yeast extract, 2% tryptone, 10 mM NaCl, 2.5 mM KCl, 10 mM MgCl<sub>2</sub>, 20 mM MgSO<sub>4</sub>, and 20 mM glucose.)
- 

- 6** Prepare the competent cells as follows:
- ▶ For each transformation reaction, place one vial (e.g. Falcon No. 2059) on ice.
  - ▶ Thaw the competent *E. coli* cells.
    - ! *Do not use cya- cells for the transformation. Such cells do not produce the lethal CAP protein, and therefore do not allow positive selection of transformed cells.*
  - ▶ Aliquot 40 µl of thawed competent cells to each of the pre-cooled vials.
  - ▶ To each vial, add 0.7 µl of 1.42 M β-mercaptoethanol.
    - ! *Commercial preparations of β-mercaptoethanol are 14.2 M. Dilute this 1:10 with water.*
  - ▶ Gently mix the components of the vials (manually).
    - ! *DO NOT use a pipette to mix the components.*
  - ▶ Incubate vials on ice for 10 min.
- 

- 7** Pipette 2 µl of the ligated DNA (ligation reaction product from Step 4) directly into the vial containing the prepared cells (from Step 6), then do the following:
- ▶ Gently mix the ligated DNA and the competent cells (manually).
    - ! *DO NOT use a pipette to mix the components.*
  - ▶ Incubate the vials on ice for 30 min.
  - ▶ Transfer the vials to a 42°C water bath and incubate for exactly 45 s.
    - ! *DO NOT mix or shake!*
  - ▶ Transfer the vials quickly to ice and incubate for 2 min.
- 

- 8** Allow each vial of transformed cells to grow as follows:
- ▶ Add 360 µl pre-warmed SOC medium (from Step 5) to the vial containing the transformed cells.
  - ▶ Place the vial in a shaking incubator and shake (220 rpm) for 30 min at 37°C.
- 

- 9** Plate the transformed cells on selective media:
- ▶ Spread 50 – 100 µl of cell suspension from each vial onto an LB agar plate containing 100 µg/ml ampicillin.
  - ▶ Invert the plates and incubate them at 37°C overnight.
- 

- 10** Analyze each colony by either:
- ▶ Performing direct colony PCR (as described in Chapter 8 in this manual).
  - ▶ Isolating the plasmid from the colony, then digesting it with restriction enzymes.
- 



## 6.2.4 Procedure for Cloning Long (7– 36 kb) PCR Products with the Expand Cloning Kit

This procedure describes cloning of long PCR products that have one-base overhangs at their 3' ends (e.g., those generated with the Expand PCR Systems). It uses components of the Expand Cloning Kit.



For details of this procedure, see the package insert for the Expand Cloning Kit (Cat. No. 11 940 392 001), which is available online at [www.roche-applied-science.com](http://www.roche-applied-science.com).



Several days before starting this procedure, do the following:

- ▶ Purchase the HIGH PURE PCR Template Purification Kit (Cat. No. 11 796 828 001) and familiarize yourself with the purification procedure described in the package insert for the kit.



The package insert for the kit is available online at [www.roche-applied-science.com](http://www.roche-applied-science.com).

- ▶ From 1 to 5 days before performing the packaging step (Step 7 below), streak the *E. coli* DH5-alpha culture (kit vial 12) onto an LB agar plate and allow the colonies to grow overnight at 37°C. These cells will be needed in Step 8 of the procedure.

- 1 Purify the long PCR product with the HIGH PURE PCR Template Purification Kit as follows:
  - ▶ Add to the PCR product an equal volume of binding buffer (green topped vial from purification kit).
  - ▶ Add 0.25 volume of isopropanol to the above mix and mix carefully.
  - ▶ Starting with Step 3 of the total nucleic acid protocol, purify the PCR product as described in the package insert of the HIGH PURE PCR Template Purification Kit.
- 2 To simultaneously polish the 3' ends and phosphorylate the 5' ends of the DNA, do the following:
  - ▶ Reduce the volume of the purified PCR product (from Step 1) to approx. 20 µl by either using a speed vac or by ethanol precipitation and resuspension.
  - ▶ To a sterile 1.5 ml reaction tube, add the following components in the order listed:

Component	Vial (from the Kit)	Volume
PCR product, purified and concentrated		approx. 20 µl
T4 DNA Polymerase reaction buffer, 5× conc.	8	8 µl
Phosphorylation buffer, 10× conc.	9	4 µl
T4 DNA Polymerase, 1 unit/µl	5	1 µl
T4 Polynucleotide Kinase, 10 units/µl	6	1 µl
Sterile, double distilled water		approx. 6 µl
<b>Total volume</b>		<b>40 µl</b>

<sup>a)</sup> Numbered vials are components of the Expand Cloning Kit.

- ▶ Incubate the tube for 20 min at 37°C.



- 3** Repeat Step 1 to purify the modified PCR product (with the HIGH PURE PCR Template Purification Kit).

 *This step is necessary to remove enzymes and reaction buffer, which can interfere with ligation and transformation.*

- 4** Adjust the concentration of the purified, modified PCR fragment to 20 – 40 ng/μl.

- 5** Depending upon the size of the modified PCR fragment, select the correct cloning vector, as follows:

If the PCR product is...	Then use...
between 7.0 and 16.5 kb	Expand Vector I (kit vial 1)
between 16.5 and 25.0 kb	Expand Vector II (kit vial 2)
between 25.0 and 36.0 kb	Expand Vector III (kit vial 3)

- 6** To ligate the purified, modified PCR fragment into the chosen Expand Vector, do the following:

▶ To a sterile 1.5 ml reaction tube, add the following components in the order listed:

Component	Volume
Purified, modified PCR product, 20– 40 ng/μl (from Step 4)	y μl <sup>a)</sup>
Expand Vector (I, II, or III), 50 ng/μl (from Step 5)	z μl <sup>a)</sup>
Sterile, double distilled water	Variable, to make final volume = 10 μl
<b>Total volume</b>	<b>10 μl</b>

<sup>a)</sup> Adjust the volumes of the vector and the PCR product such that (1) the molar ratio of the vector to the product is 1:2, and (2) the total amount of DNA in the reaction is between 200 and 300 ng.

- ▶ Mix the contents of kit vial 10 (ligation buffer<sup>PLUS</sup>) thoroughly.
- ▶ To the reaction tube containing PCR product and vector, add 10 μl ligation buffer<sup>PLUS</sup> and mix carefully.
- ▶ Add 1 μl T4 DNA ligase (5 units/μl, kit vial 7).
- ▶ Mix contents of tube carefully (manually), then centrifuge briefly.
- ▶ Incubate the tube for 30 min at 15 to 25°C.
- ▶ Incubate the tube for 5 min at –60°C (or below).

- 7** To package the ligated DNA in lambda phage heads, do the following:

▶ For each sample of ligated DNA, thaw one tube of DNA packaging extract (kit vial 11) on ice.

 *For best results, always thaw the packaging extract on ice. Once the packaging extract is thawed, it must either be used or thrown away. Thawed extract does not “keep” for very long.*

- ▶ Add 5 μl of the ligation reaction mix (from Step 6) to the tube containing the DNA packaging extract.
- ▶ Mix the contents of the tube carefully with a pipette.
- ▶ Centrifuge the tube briefly.
- ▶ Incubate the tube for 2 h at 15° to 25°C.
- ▶ To the tube, add 300 μl of SM phage dilution buffer and 20 μl chloroform.

 *SM phage dilution buffer contains 0.1 M NaCl, 0.01 M MgSO<sub>4</sub> • 7H<sub>2</sub>O, 0.05 M Tris-HCl (pH 7.5), and 0.01% gelatin.*

- ▶ Mix carefully and centrifuge the mixture briefly.
- ▶ Store the phage supernatant at +2 to +8°C until used (Step 9).



- 
- 8** Prepare an *E. coli* magnesium culture as follows:
- ▶ To a 100 ml flask, add 20 ml LB medium containing 10 mM MgSO<sub>4</sub> and 0.2% (w/v) maltose.
  - ▶ Inoculate the medium with a 1 – 5 day old colony of *E. coli* DH5-alpha from an LB agar plate.
  - ▶ Shake the flask at 37°C until the cells have reached an absorbance (A<sub>600</sub>) of 0.8– 1.0 (about 3 – 4 h).
  - ▶ Harvest the bacteria by centrifuging for 5 min at 3500 rpm and +2 to +8°C.
  - ▶ Resuspend the bacterial pellet in 10 mM MgSO<sub>4</sub> and adjust the absorbance (A<sub>600</sub>) of the suspension until it is between 0.8 and 1.0.
- 
- 9** Infect the *E. coli* magnesium culture (from Step 8) with the phage supernatant (from Step 7) as follows:
- ▶ In sterile 2 ml reaction tubes, prepare two different mixes of phage supernatant and *E. coli* magnesium culture:
  - ▶ Mix A: 50 µl phage supernatant + 100 µl *E. coli* magnesium culture
  - ▶ Mix B: 200 µl phage supernatant + 400 µl *E. coli* magnesium culture
  - ▶ Incubate mix A and mix B for 30 min at 15° to 25°C.
  - ▶ Add 1.0 ml LB medium to each mix.
  - ▶ Incubate mix A and mix B for 60 min at 37°C, swirling the contents of the tubes carefully every 15 min during the incubation.
  - ▶ Centrifuge the tubes for 3 min at 13,000 rpm and discard the supernatant.
  - ▶ Resuspend the pellet in each tube carefully in 50 µl fresh LB medium.
- 
- 10** Plate the cells from each suspension on a separate LB plate containing 100 µg/ml ampicillin, then incubate the plates overnight at 37°C.
- 
- 11**
- ▶ Isolate the cosmids from the *E. coli* colonies.
  - ▶ Analyze the cloned DNA by standard methods (Sambrook and Russell, 2001).
-  For suggestions on preparing and analyzing the DNA, see the package insert for the Expand Cloning Kit (Cat. No. 11 940 392 001), which is available online at [www.roche-applied-science.com](http://www.roche-applied-science.com).
-

## 6.2.5 Activity of Restriction Enzymes in Standard Taq DNA Polymerase Buffer

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For various reasons, including direct cloning of amplified DNA, researchers want to add restriction enzyme directly to a PCR reaction mix and digest the PCR product without first purifying it. We have investigated 71 restriction enzymes to determine which enzymes show sufficient activity to be used directly in a standard Taq DNA Polymerase reaction buffer.

For each experiment, a standard PCR was performed, with lambda DNA as template, in a 100 µl reaction mix that contained primers, 10 mM Tris-HCl (pH 8.3, 20°C), 50 mM KCl, 1.5 mM MgCl<sub>2</sub>, 200 µM dNTPs and 2.5 units Taq DNA Polymerase. The mix was amplified for 25 cycles. After the reaction, the restriction enzyme and its substrate DNA (*i.e.*, the substrate normally used to determine the activity of the enzyme) were added to the PCR mix and the activity of the enzyme was determined by standard procedures. The activity of the restriction enzyme in the PCR mix was compared to the activity of the same enzyme under optimal reaction conditions. The results are shown in Table 6.2.1.

**Table 6.2.1: Activity of Restriction Enzymes in Taq DNA Polymerase PCR Mix.**

RE	% Activity <sup>a</sup>	RE	% Activity <sup>a</sup>	RE	% Activity <sup>a</sup>	RE	% Activity <sup>a</sup>
<i>Aat</i> II	25	<i>Bst</i> EII <sup>b</sup>	100	<i>Kpn</i> I	50	<i>Pvu</i> II	100
<i>Acc</i> I	<5	<i>Bst</i> XI	0	<i>Ksp</i> I	0	<i>Rsa</i> I	100
<i>Acy</i> I	0	<i>Cfo</i> I	100	<i>Mae</i> I	0	<i>Sac</i> I	100
<i>Afl</i> III	20	<i>Cla</i> I	100	<i>Mae</i> II	0	<i>Sal</i> I	0
<i>Alu</i> I	100	<i>Dde</i> I	40	<i>Mae</i> III	0	<i>Sau</i> 3A	100
<i>Apa</i> I	100	<i>Dpn</i> I	100	<i>Mam</i> I	20	<i>Sca</i> I	<5
<i>Asp</i> 700	10	<i>Dra</i> I	100	<i>Mlu</i> I	<5	<i>Sfi</i> I	10
<i>Asp</i> 718	100	<i>Dra</i> III	50	<i>Msp</i> I	40	<i>Sma</i> I	100
<i>Ava</i> I	20	<i>Ecl</i> XI	0	<i>Mvn</i> I	30	<i>Sna</i> BI	50
<i>Ava</i> II	<5	<i>Eco</i> 47 III	0	<i>Nae</i> I	0	<i>Sph</i> I	<5
<i>Avi</i> II	30	<i>Eco</i> RI	50	<i>Nco</i> I	50	<i>Ssp</i> I	0
<i>Bam</i> HI	100	<i>Eco</i> RV	10	<i>Nde</i> I	0	<i>Stu</i> I	30
<i>Bbr</i> PI	100	<i>Hae</i> III	100	<i>Nhe</i> I	100	<i>Sty</i> I	<5
<i>Bfr</i> I	100	<i>Hind</i> II	100	<i>Not</i> I	0	<i>Taq</i> I	100
<i>Bgl</i> I	30	<i>Hind</i> III	10	<i>Nru</i> I	75	<i>Xba</i> I	60
<i>Bgl</i> II	0	<i>Hinf</i> I	50	<i>Nsi</i> I	100	<i>Xho</i> I	<5
<i>Bsm</i> I	0	<i>Hpa</i> I	100	<i>Pst</i> I	90	<i>Xma</i> CI	0
<i>Bss</i> HII	100	<i>Hpa</i> II	40	<i>Pvu</i> I	<5		

<sup>a)</sup> Activity is expressed as a percentage of the activity of the enzyme under optimal salt and buffer conditions (*i.e.*, the standard activity assay in the appropriate incubation buffer supplied with the restriction enzyme).

<sup>b)</sup> Restriction enzyme *Bst* EII shows increased activity on lambda DNA



## 6.2.6 Activity of Restriction Enzymes in Pwo SuperYield DNA Polymerase Buffer

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To facilitate downstream applications such as direct cloning of amplified DNA, it would be convenient to perform a restriction enzyme digest directly in the PCR mix, without first purifying the amplified product. To find restriction enzymes that show sufficient activity for such direct use, we tested the activity of 22 restriction enzymes in two PCR mixes with Pwo SuperYield DNA Polymerase.

For each experiment, one PCR mix, prepared as recommended in the package insert for the polymerase, contained Pwo SuperYield DNA Polymerase, dNTPs, primers specific for the human tPA gene and PCR Grade water. A second PCR mix was identical, except that it also contained the GC-RICH Solution supplied with the polymerase. As template, the mixes contained either lambda DNA or pBR322 plasmid (for the *Dpn* I and *Sal* I experiments). After PCR, the appropriate restriction enzyme was added to each mix and the activity was determined by standard procedures. The activity of the restriction enzyme in the PCR mix was compared to the activity of the same enzyme under optimal reaction conditions. The results are shown in Table 6.2.2.



**Table 6.2.2: Activity of Restriction Enzymes in Pwo SuperYield PCR Mix.**

RE	Recommended SureCut Buffer	Relative Activity (%) in PCR Mix 1 <sup>a</sup>	Relative Activity (%) in PCR Mix 2 <sup>b</sup>
<i>Apa</i> I	A	10	>100
<i>Bam</i> HI	B	100	>100
<i>Bgl</i> II	M	85	100
<i>Cla</i> I	H	>100	>100
<i>Dpn</i> I <sup>c,d</sup>	A	100	100
<i>Eco</i> RI	H	100	10 <sup>e</sup>
<i>Eco</i> RV	B	25	25
<i>Hind</i> III	B	25	25
<i>Kpn</i> I	L	100	100
<i>Nco</i> I	H	100	50
<i>Nde</i> I	H	40	100
<i>Nhe</i> I	M	>100	>100
<i>Not</i> I	H	25	15
<i>Nru</i> I	B	10	25
<i>Pst</i> I	H	35	15
<i>Sac</i> I	A	100	20
<i>Sal</i> I <sup>d</sup>	H	<10	<10
<i>Sma</i> I	A	>100	100
<i>Sph</i> I	M	30	5 - 10
<i>Xba</i> I	H	25	100
<i>Xho</i> I	H	15	15
<i>Xma</i> CI	L	0	<5

- a) PCR Mix contained lambda DNA template, Pwo SuperYield DNA Polymerase, dNTPs, primers and buffer.  
b) PCR Mix contained lambda DNA template, Pwo SuperYield DNA Polymerase, dNTPs, primers, buffer and GC-RICH Solution.  
c) Enzyme requires methylated DNA.  
d) pBR322 was used as template.  
e) Increased star activity was detected.



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# 6

*Chapter 7*

# Real-Time PCR Methods



<b>7</b>	<b>Real-Time PCR Methods</b>	<b>Page</b>
7.1	Introduction .....	195
7.2	Real-Time PCR Assay Formats .....	198
7.3	Quantification Methods for Real-Time PCR .....	203
7.4	Product Characterization and Genotyping by Melting Curve Analysis .....	207
7.5	Real-Time PCR Instruments Available from Roche Applied Science.....	211
7.6	Real-Time PCR Reagents.....	215
7.7	Published Examples of Applications for the LightCycler® Carousel-Based Systems.....	224



## 7. Real-Time PCR Methods

### 7.1 Introduction

In most of the articles in this PCR Applications Manual, PCR products are analyzed in a separate procedure, which is performed after PCR is completed. We call this type of analysis “end-point analysis” (because it is usually done after 30–40 cycles of PCR). It is routinely used for qualitative analysis, but can also be used for semi-quantitative analysis (e.g., competitive PCR). Gel electrophoresis is usually used to assess the presence or absence of particular products, as well as the size and purity of the products. Ethidium bromide is the fluorescent dye most commonly used to visualize the products on the gel. For quantification in end-point analysis, the products are usually transferred to a blot and analyzed with labeled probes.

End-point analysis is not very suitable for quantitative PCR because it is done in the “plateau phase” of PCR where the reaction no longer follows exponential kinetics. In this phase, the reaction can no longer be described by a mathematical formula. Thus, it is not possible to directly correlate the end-point signal with the initial template amount or target copy number. Further, in the plateau phase, PCR efficiency decreases steadily, because reaction compounds are being consumed and inhibitors are accumulating. These effects vary from sample to sample, resulting in different end-point signals.

Real-time PCR offers an alternative method for both qualitative and quantitative analysis. This type of analysis allows the amplification and fluorescent detection steps to be performed by a single instrument in a single tube with data recorded online. A real-time PCR instrument measures the accumulation of PCR products during amplification with fluorescent dyes. Because PCR itself and the detection of PCR products occur in the same reaction (vessel), this set-up is also called “homogeneous PCR”.

#### Advantages of Real-Time PCR for Qualitative Real-Time Analysis

Many important PCR applications require only a qualitative answer, that is, whether a particular target sequence is present or absent in a particular sample. Real-time PCR has several important advantages over end-point analysis for qualitative determinations:

- ▶ Real-time PCR is much faster than end-point methods because the time-consuming separate analysis step is eliminated. Not only that, but the “Yes-No” answer can be obtained much more quickly (*i.e.*, during the exponential phase), rather than after the amplification is complete.
- ▶ Real-time PCR produces very reliable results because the sophisticated detection instrument in real-time instruments is far more sensitive than ethidium bromide staining.
- ▶ Real-time PCR minimizes the chance of contamination since both amplification and detection take place in a single closed tube.



### Advantages of Real-Time PCR for Quantitative Real-Time Analysis

Sophisticated real-time PCR instrumentation and reagents have turned PCR into an effective tool for quantification, one with unsurpassed sensitivity and much greater linear range than blotting procedures. To explain why this is true, we have to briefly describe how real-time fluorescent analysis of the PCR cycle works.

#### Real-Time Fluorescent Analysis of a PCR Cycle

If PCR products are fluorescent and fluorescence is plotted against cycle number, the accumulation of PCR products can be shown as a curve, similar to a bacterial growth curve. This amplification curve has three segments (Figure 7.1.1): an early background phase (below the detection level of the instrument), an exponential growth phase (or log phase) and a plateau (end-point) phase. The background phase lasts until the signal from the PCR product is greater than the background signal of the system. The exponential growth phase begins when sufficient product has accumulated to be detected above background, and ends when the reaction efficiency falls (as the reaction enters the plateau phase).

During the exponential phase, the reaction can be described mathematically as:

$$N_n = N_0 \times (E_{\text{const}})^n$$

( $N_n$ : number of molecules at  $n$  cycle;  $N_0$ : initial number of molecules;  $E_{\text{const}}$ : constant amplification efficiency;  $n$ : number of cycles)

Usually only 4-6 cycles from a 40-cycle PCR show this exponential growth. After that, the reaction components become limiting. During the exponential phase, the initial number of target molecules ( $N_0$ ) can be calculated from the above equation.

In the plateau phase of PCR, efficiency becomes variable and amplification is best expressed as:

$$N = N_0 \times (E_{\text{var}})^n.$$

Because the  $E_{\text{var}}$  value is not known (in contrast to the  $E_{\text{const}}$  value), it is not possible to calculate  $N_0$  during the plateau phase as it is during the log phase.

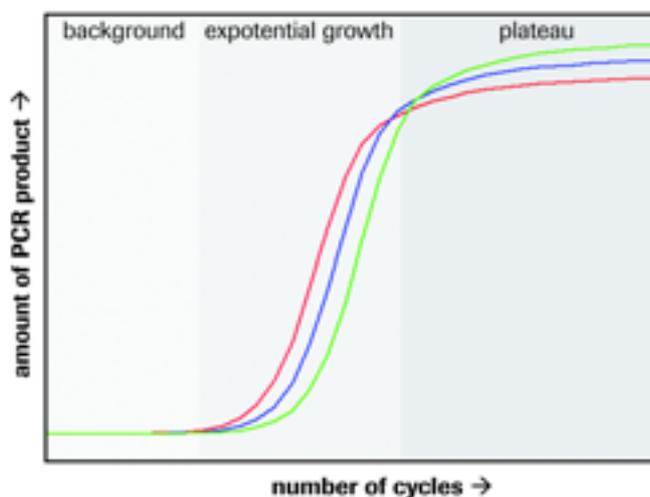


Figure 7.1.1. Typical amplification curves

### How Real-Time PCR Simplifies Quantitative Analysis

In real-time PCR, data on PCR product concentration is collected online from every cycle. The real-time PCR instrument can display this data as an amplification curve (like Figure 7.1.1) or in other graphical formats. After the reaction is over, the investigator can easily identify the exponential cycles by simply examining the record of the run. Then, the data can be used to determine the amount of PCR product present during the exponential phase and, by extrapolation, the initial amount of template in each reaction. Sophisticated real-time PCR instrumentation and software make quantitative analysis of this exponential phase data both possible and simple.

### Drawbacks of End-Point Analysis for Quantitative PCR

In contrast, with traditional end-point PCR methods, quantification during PCR is nearly impossible.

In end-point analysis, a fluorescent signal is usually obtained after a run is completed (*i.e.*, by fluorescent analysis of the PCR product during the plateau phase). Yet, the amount of PCR product that accumulates in the plateau phase (and generates the fluorescence signal) rarely correlates with the initial template amount.

Figure 7.1.1 (above) illustrates this drawback. In that figure, although the “green” sample contains a lower initial amount of target than the “red” and “blue” samples, it produces the highest plateau phase signal. This most probably occurs because the “green” reaction has a higher PCR efficiency than the “red” and “blue” reactions.

The most accurate quantitative determinations can only be made during the exponential phase of the PCR. However, with conventional PCR, finding the 4-6 exponential cycles out of 40 is at best tedious and at worst almost impossible. This usually requires sampling the PCR repeatedly, then analyzing the multiple samples. The only alternative would be to know which cycles were exponential from the very beginning, a daunting task to say the least.

Such PCR methods as competitive PCR or limiting dilution PCR can be used to determine the amount of PCR products after a run is completed. However, the accuracy of the determination depends on the sensitivity of the detection method (such as blotting) and the ability of the investigator to take the product through multiple handling steps without losing any.



## 7.2 Real-Time PCR Assay Formats

All real-time PCR systems detect a fluorescent dye, and then correlate this fluorescence signal to the amount of PCR product in the reaction. There are several methods for detecting and evaluating PCR products fluorimetrically. The most commonly used fluorescent assay methods fall into two classes, sequence-independent detection assays and sequence-specific probe binding assays. Each has its uses and its limitations.

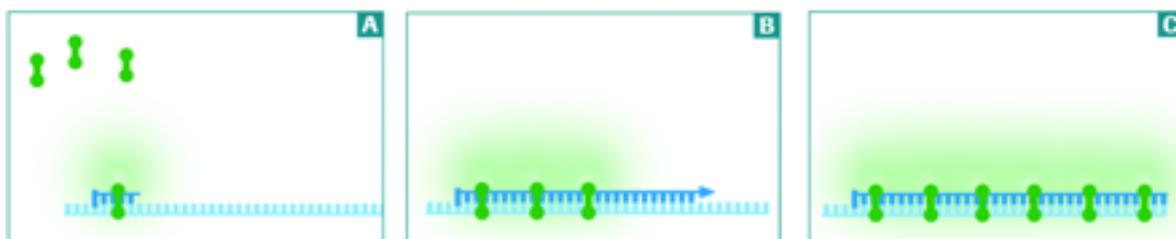


For more detailed and up-to-date descriptions of real-time PCR detection formats visit [www.lightcycler.com](http://www.lightcycler.com).

### Sequence-Independent Detection Assays

Sequence-independent assays rely on fluorophores (typically SYBR Green I) that bind to all double-stranded DNA molecules regardless of sequence.

SYBR Green I barely fluoresces (Figure 7.2.1) when it is free in solution, but its fluorescence emission is greatly enhanced when it binds to double-stranded DNA (due to conformational changes in the dye). Thus, the increase in SYBR Green I signal (measured at 530 nm) correlates with the amount of product amplified during PCR. For optimal results, the fluorescence signal (at 530 nm) should be acquired (measured) at the end of each elongation phase. Increases in PCR product can then be monitored from cycle to cycle. The major advantage of the SYBR Green I assays is that they are relatively easy to design and optimize. All you need to do is design a set of PCR primers and experimentally optimize the amplification efficiency and specificity. Thus SYBR Green I provides a simple, convenient way to qualitatively detect or quantify any PCR or RT-PCR product.



**Figure 7.2.1. PCR in the presence of SYBR Green I.** SYBR Green I dye only fluoresces when it is bound to double-stranded DNA (dsDNA) and excited by blue light. SYBR Green I does not bind to single-stranded DNA, so fluorescence is minimal during denaturation. As dsDNA forms (**panel A**) and is synthesized (**panel B**), SYBR Green I binds the dsDNA and the fluorescent signal from the bound SYBR Green I (green light) increases. At the end of elongation (**panel C**), all DNA is double-stranded, the maximum amount of SYBR Green I is bound and the fluorescent signal is at its highest for that PCR cycle. Therefore, the fluorescent signals from SYBR Green I are measured at the end of each elongation phase.

However, since SYBR Green I binds to any dsDNA, the SYBR Green I format cannot discriminate between different dsDNA species. Specific product, nonspecific products and primer-dimers are all detected equally well. Since any double-stranded PCR artifact will contribute to signal intensity, the assay may overestimate the actual concentration of the target sequence.

To get around this problem, the assay can be extended to include a melting curve analysis. This analysis (subsequent to the PCR run) can quickly discriminate between specific product, primer-dimer and any other artifacts, based on their specific melting temperatures. Thus, a melting curve determination should always be included in any qualitative run that uses SYBR Green I for detection.



See section 7.4 for details on melting curve analysis.

### Sequence-Specific Probe Binding Assays

Sequence-specific assays rely on oligonucleotide probes that hybridize to their complementary sequence in the target PCR product and thus only detect this specific product. Commonly used probe formats are hydrolysis probes, hybridization probes (e.g., **HybPr>be** probes) or single-labeled probes (e.g., **SimplePr>be** probes). The probes are coupled to fluorophores that can be measured by the real-time PCR instrument. Several other detection formats are available as well, but are less frequently used.



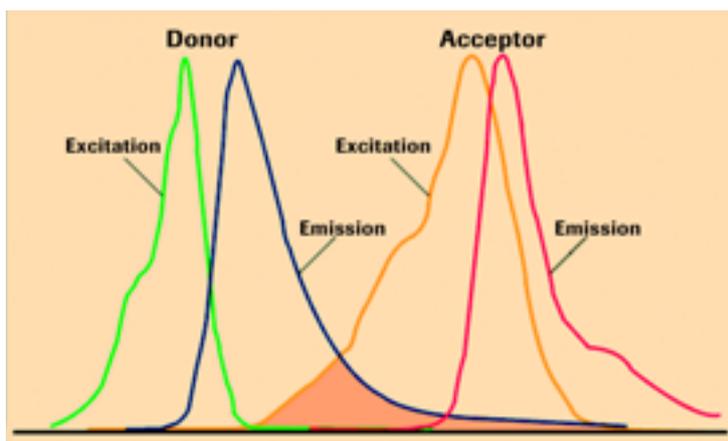
The main application of single-labeled (*SimpleProbe*) probes is mutation detection. This format is described in more detail in section 7.4.

A PCR assay that uses fluorophore-labeled, sequence-specific probes can be highly specific, since assay fluorescence increases only if the specific target is present in the reaction. Due to this sequence specificity, artificial by-products (such as primer-dimers or PCR by-products) will not be detected. A separate melting curve analysis to differentiate product from nonspecific artifact is not required.

### FRET Principle

Most sequence-specific probe formats are based on the so-called *FRET* principle. *Fluorescence Resonance Energy Transfer* (FRET) is based on the transfer of energy from one fluorescent molecule (e.g., fluorescein) to another adjacent fluorescent molecule (e.g., LightCycler® Red 640) (Figure 7.2.2). For example, if fluorescein is excited by blue light, it can either emit fluorescent light of wavelength 530 nm or transfer this energy to a LightCycler® Red 640 molecule (which is not directly affected by the blue light). Then the LightCycler® Red 640 molecule will in turn be excited and the light emitted can be measured at 640 nm.

# 7



**Figure 7.2.2. FRET process.** A photon from a light source excites a donor. The energy absorbed by the donor is subsequently transferred and absorbed by a nearby acceptor molecule that has an overlapping spectrum. The acceptor, in turn, emits light that can be measured at a longer wavelength. When the energy is released, all electrons return to ground level, ready for another excitation/emission cycle.

Primary prerequisites for FRET are:

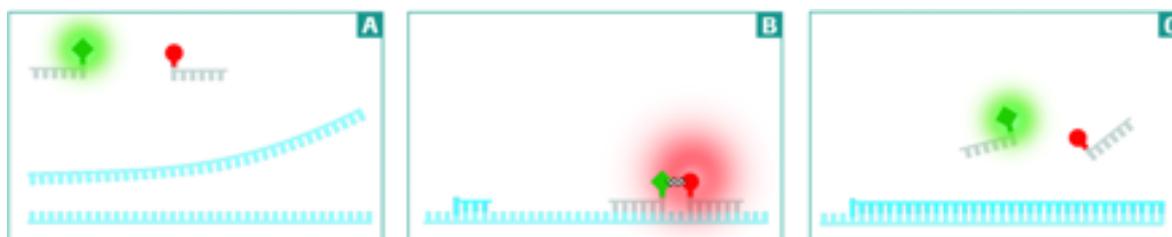
- ▶ Donor and acceptor molecules must be close to each other.
- ▶ The excitation spectrum of the acceptor must overlap the fluorescence emission spectrum of the donor (Figure 7.2.2).
- ▶ The dipole orientations of donor and acceptor must be approximately parallel.

This FRET process can be used in various ways to generate a sequence-specific signal during PCR. While the hydrolysis probe format is based on quenching the fluorescence of the donor dye, **HybProbe** probes rely on the fluorescence emission of the acceptor dye. In both formats, the two fluorophores must have the characteristics described above.

### HybProbe Format

The HybProbe format is suitable for both quantitative PCR and mutation (SNP) detection assays. It uses two specially designed oligonucleotides that hybridize, side by side, to an internal sequence of an amplified fragment during the annealing phase of PCR. Both probe molecules are labeled with different dyes (the donor and the acceptor dye of the FRET process); the upstream probe is labeled at the 3' end, the downstream probe at the 5' end. (The probe labeled at the 5' end must also be phosphorylated at its 3' end to keep it from being extended during PCR.) The energy of the donor dye from the first probe can excite the acceptor dye on the second HybProbe probe, which then emits fluorescent light at a different wavelength. The instrument detects this emitted light.

The energy transfer from the donor to the acceptor depends greatly on the spacing between the two dye molecules. Energy is only transferred efficiently if the molecules are very close to each other (between 1 and 5 nucleotides). These two fluorescent labels are close to each other only when the two oligonucleotides anneal to adjacent regions on the target, which makes FRET possible (Figure 7.2.3). Therefore, the amount of fluorescence emitted is directly proportional to the amount of target DNA generated during PCR.



**Figure 7.2.3. Analysis with HybProbe probes.** Panels A-C show the behavior of HybProbe probes during different stages of PCR. In the example shown, the donor dye probe has a fluorescein label at its 3' end, and the acceptor dye probe has a LightCycler® Red label at its 5' end. (Note that this set-up might also be reversed, *i.e.*, donor dye at the 5' end and acceptor dye at the 3' end.) Hybridization does not occur during the denaturation phase of PCR (**panel A**). Since the distance between the unbound dyes prevents energy transfer, no fluorescence will be emitted by the red acceptor dye during this phase. In **panel B**, the probes hybridize to the amplified DNA fragment in a head-to-tail arrangement, thereby bringing the two fluorescent dyes close to each other. Fluorescein is excited by blue light, which causes it to emit green fluorescent light. The emitted energy excites the LightCycler® Red dye. After annealing, an increase in temperature leads to elongation and displacement of the probes. At the end of the elongation step, the PCR product is double-stranded, while the displaced HybProbe probes are back in solution and too far apart to allow FRET to occur (**panel C**). The red fluorescence emitted by the second probe is measured at the end of each annealing step (**panel B**), when the fluorescence intensity is greatest.

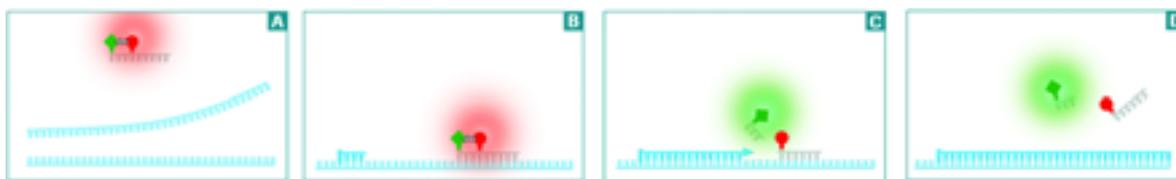


*One of the advantages of the HybProbe detection format is that the probes are not altered during PCR. At the end of amplification, both probes are still intact and may be used in a subsequent melting curve experiment (e.g., for mutation detection or SNP analysis).*

### Hydrolysis Probe Format

The strength of the hydrolysis probe format is its performance in quantitative PCR assays. Hydrolysis probes emit fluorescence when the 5' exonuclease activity of Taq DNA Polymerase hydrolyzes them (Figure 7.2.4). These assays are actually homogeneous 5' nuclease assays, since the assay uses cleavage of a single 3'-non-extendable probe to indicate the accumulation of a specific target DNA sequence. This single probe contains both a fluorescence reporter and a fluorescence quencher, which are close to each other. When the probe is intact, the quencher dye is close enough to the reporter dye to suppress the reporter fluorescence signal (fluorescence quenching via FRET). During PCR, the 5' nuclease activity of the polymerase cleaves the probe, separating the reporter and quencher, permitting the reporter dye to emit fluorescence.

# 7



**Figure 7.2.4. Analysis with hydrolysis probes.** Panels A-D show the behavior of hydrolysis probes during PCR. The probe carries two fluorescent dyes in close proximity, one of which (quencher) quenches the fluorescence from the other (reporter) as long as the probe is intact. In the denaturation phase (**panel A**), the strands of the target DNA separate as the temperature increases. During the annealing phase (**panel B**), primers and probes specifically anneal to the target sequence. (The hydrolysis probe is phosphorylated at the 3' end, so it cannot be extended.) As the DNA polymerase extends the primer, the 5' nuclease activity of the enzyme will cleave the probe (**panel C**), allowing the reporter dye to emit green fluorescence. The probe fragments are then displaced from the target, and polymerization of the new amplicon continues (**panel D**). The DNA polymerase will separate the reporter and quencher only if the probe has hybridized to the target. Accumulation of PCR products is detected directly by monitoring the increase in green fluorescence from the reporter dye. The fluorescent signal of the reporter dye is measured at the end of each elongation phase (**panel C**).



Unlike **HybProbe** probes, hydrolysis probes are digested during PCR. Thus, these probes cannot be used in a subsequent melting curve experiment. This type of assay requires a different experimental approach for detecting a mutation or SNP. Roche Applied Science will offer hydrolysis probe based genotyping in combination with the LightCycler® 480 System in future. Please visit [www.lightcycler.com](http://www.lightcycler.com) for latest information.

### Universal ProbeLibrary

A sophisticated application of the hydrolysis probe format is the Universal ProbeLibrary from Roche Applied Science. The Universal ProbeLibrary contains 165 prevalidated, double-labeled, real-time PCR probes that can be used to quantify virtually any transcript in the transcriptomes of humans, mice, rats, primates, *Drosophila*, *C. elegans*, and *Arabidopsis*.

Each of the organism-specific Universal ProbeLibrary Sets can detect 95-99% of all transcripts from that organism. This almost universal coverage is due to the length (only 8-9 nucleotides) of the Universal ProbeLibrary probes. Each probe is much shorter than classic, 25- to 35-nucleotide hydrolysis probes. To maintain the specificity,  $T_m$  and assay compatibility that hybridization probes require, the duplex-stabilizing DNA analogue LNA (Locked Nucleic Acid) is included in the sequence of each probe.

Because it is short, each Universal ProbeLibrary probe can bind to around 7,000 transcripts, while each transcript is detected by around 16 different probes. Yet, only one transcript is detected in a given PCR assay, a specificity ensured by the set of PCR primers chosen.

Selection of the correct Universal ProbeLibrary probe and specific PCR primers for a given real-time PCR assay is a simple two-step procedure that involves the ProbeFinder Software, which is available online at the Assay Design Center (<http://www.universalprobelibrary.com>).



Universal ProbeLibrary assays are compatible with all instruments capable of detecting fluorescein, FITC, FAM and/or SYBR Green I. Universal ProbeLibrary assays have been used successfully on the LightCycler® Carousel-Based System, the LightCycler® 480 System and other real-time PCR instruments from several suppliers.

## 7.3 Quantification Methods for Real-Time PCR

In an amplification reaction, the cycle at which the fluorescence of a sample rises above background is called the crossing point (CP) of the sample, *i.e.*, the point at which amplified product is first visible in the data. On a graph of experimental fluorescence vs. cycle number, the CP of a sample appears as a sharp, upward curve.

Quantification in real-time PCR involves determination of the CP of a sample. For PCR products to be measured by a real-time PCR instrument, the number of product molecules must exceed the detection limit of that instrument. (For example, at CP, approximately  $10^{11}$  to  $10^{12}$  product molecules are present in a LightCycler<sup>®</sup> Carousel-Based System reaction). The CP of a sample thus depends on the initial concentration of DNA in the sample; a sample with a lower initial concentration of target DNA requires more amplification cycles to reach the CP, while a sample with a higher concentration requires fewer cycles.

The software of the LightCycler<sup>®</sup> Carousel-Based System uses two different methods to determine the CP:

- ▶ An automated method, which identifies the CP of a sample as the point where the sample's fluorescence curve turns sharply upward. This turning point corresponds to the maximum of the second derivative of the curve. Thus, this method is called the "2nd Derivative Maximum Method". The great advantage of this method is that it requires little user input.
- ▶ A manual method, which determines the CP by plotting a regression line fitted to the measured fluorescence points that accumulate during the log-linear part of the PCR curve. The CP is the intersection of that regression line with a horizontal crossing line that can be set by the user. This method is called the "Fit Points" method.



*With LightCycler<sup>®</sup> 480 Software 1.2 (available July 2006), a fully automated version of the Fit Points method is introduced.*

## Two Basic Types of Quantification Analysis

Quantification analysis in real-time PCR can be subdivided into two basic types, absolute and relative quantification (Figure 7.3.1). Each type uses the experimentally determined CP values differently.

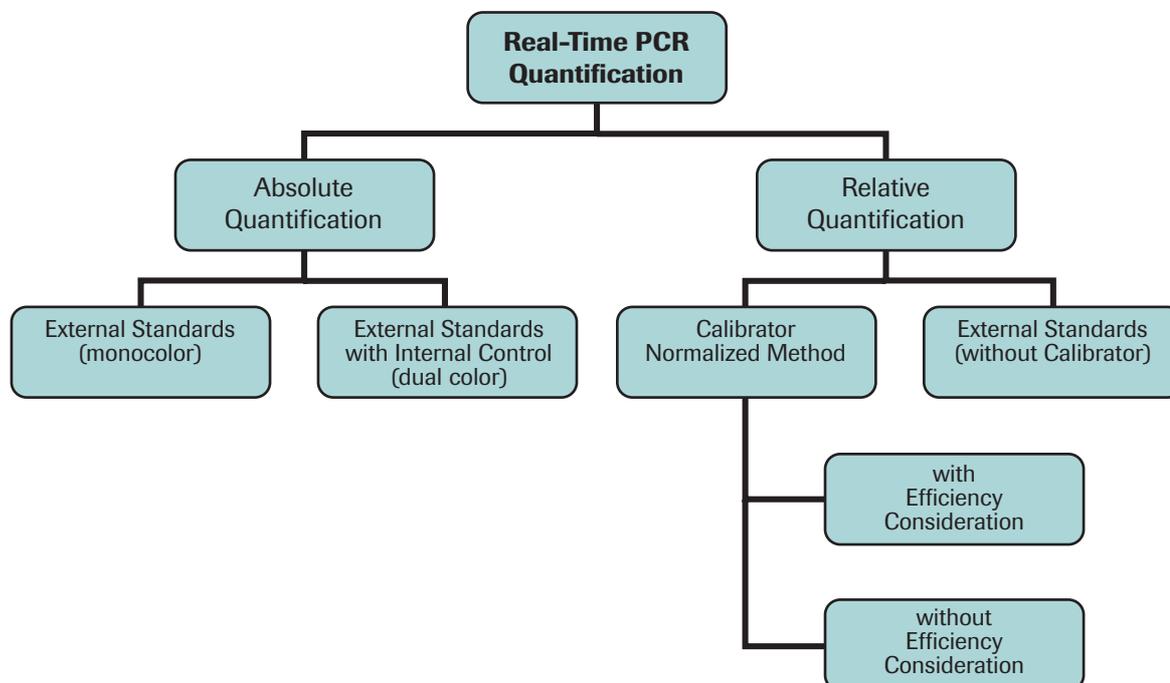


Figure 7.3.1. Real-time PCR quantification methods.

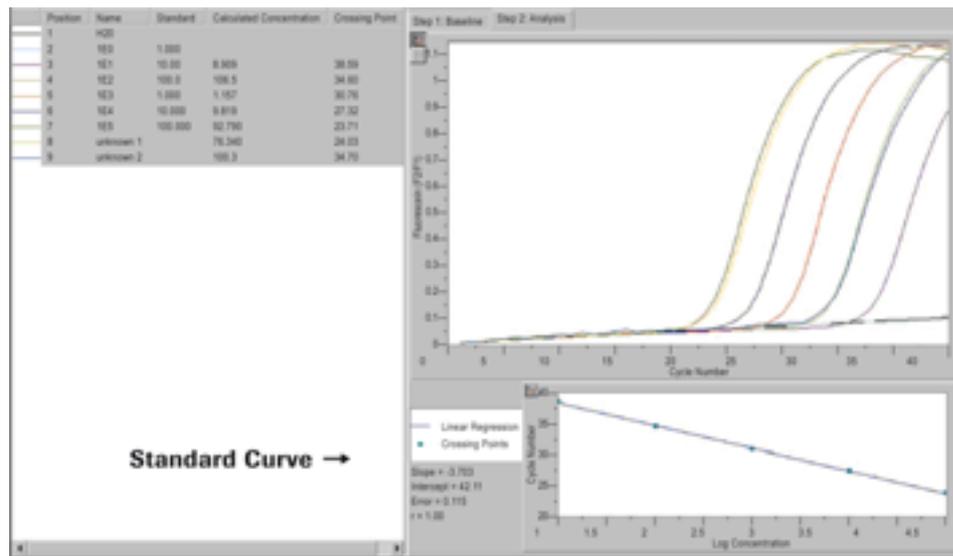
### Absolute Quantification

In absolute quantification assays, the concentration of the target molecule is expressed as an absolute value (e.g., copies,  $\mu\text{g}/\mu\text{l}$ , etc.). Absolute quantification methods use a standard curve, calculated from external standard samples of known concentration, to determine the concentration of the target molecule in the unknown. Thus, an absolute quantification assay system produces valid results only if the standards and the unknowns are amplified and detected with the same efficiency.

To generate a standard curve (Figure 7.3.2), the concentrations of standard samples are plotted against the CPs of the samples. The X-axis represents the log of the initial target concentration, and the Y-axis represents CP in cycles. The standard curve is a regression line through these plotted data points.



*The external standard samples are amplified in separate reactions but within the same real-time PCR run. The concentrations chosen for the standard curve should fall within the expected concentration range of the target in the unknowns. For greatest accuracy, the run must include enough standards to produce a statistically valid standard curve.*



**Figure 7.3.2. Standard curve derived from data generated in a HybProbe assay targeting  $\beta$ -globin (LightCycler® Software 4.x [ x stands for all software versions from 4.0 on]).**

Absolute Quantification is very suitable for applications in virology and microbiology where you need to determine the copy number of a specific target, or for the determination of absolute gene copy numbers. In a dual-color set-up, absolute quantification can be combined with internal standards to detect any false negative results.

### Relative Quantification

In relative quantification assays, the target concentration is expressed as a ratio of target-to-reference gene in the same sample, rather than an absolute value. The reference gene is an unregulated nucleic acid that is found at constant copy number in all samples.

Relative quantification methods correct the sample for differences in quality and quantity like variations in initial sample amount, cDNA synthesis efficiency, or sample loading/pipetting errors. Because the quantity of a target and a reference gene is a function only of the PCR efficiency and the sample crossing point, these assays do not require a standard curve in each analysis run.

Relative quantification assays can be refined by applying two parameters:

- ▶ Normalization of the target/reference ratios in the samples with respect to the target/reference ratio in a calibrator (which allows you to compare many different PCR experiments).

A calibrator corrects for differences in detection sensitivity between target and reference (e.g., due to differences in probe annealing, FRET efficiency, or dye extinction coefficients).

- ▶ Correction for any differences in PCR efficiencies of target and reference genes. The accuracy of the result is influenced by differences in PCR efficiency between amplification of target and amplification of reference. While a calibrator corrects for differences in detection sensitivity, it does not correct for differences in PCR efficiency between the target and reference gene. To compensate for the effects of such PCR efficiency differences requires the use of relative standard curves.

Because not every PCR assay occurs with optimal PCR efficiency ( $E = 2$ ) or with a constant PCR efficiency, we strongly recommend correcting all relative quantification assays for differences in efficiency. Only by applying such a correction to the data, you can significantly reduce calculation errors due to differences in amplification of target and reference genes.



*Relative Quantification software, which enables performance of calibrator-normalized relative quantification with PCR efficiency correction, is available for all real-time PCR systems from Roche Applied Science. (These systems are described in section 7.5.)*

Relative Quantification is very suitable for any kind of gene expression analysis or determination of relative gene dosage levels.



*For more detailed and up-to-date descriptions of quantitative real-time PCR methods, visit [www.lightcycler.com](http://www.lightcycler.com).*

## 7.4 Product Characterization and Genotyping by Melting Curve Analysis

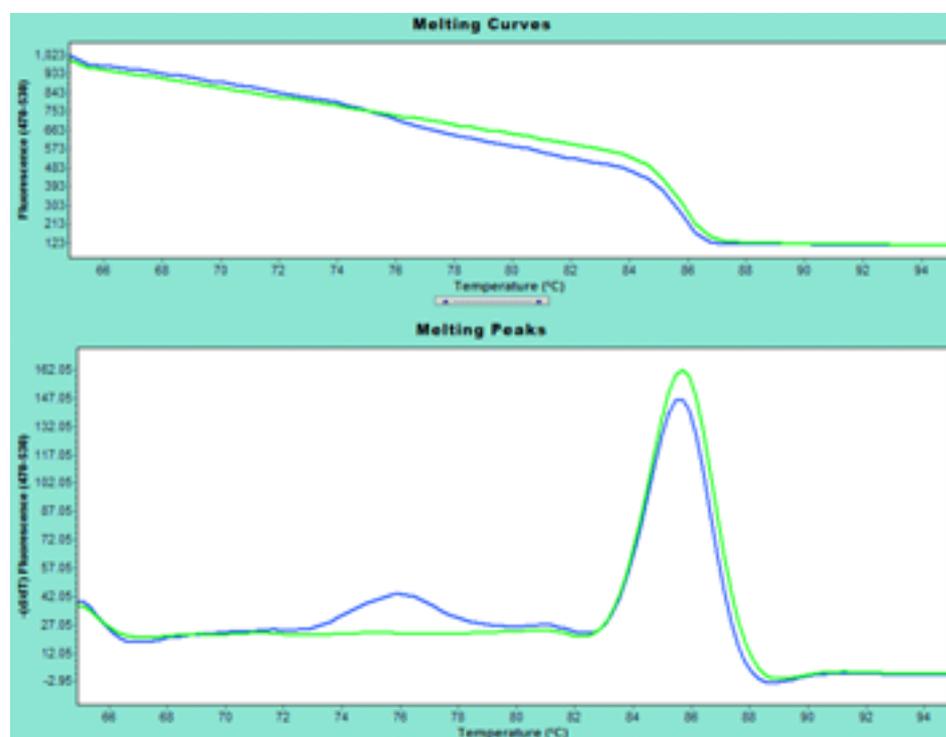
Besides monitoring the PCR process online, real-time PCR instruments (including the LightCycler® Carousel-Based System and LightCycler® 480 System) can monitor fluorescence changes during temperature transitions. This allows the annealing and denaturation of nucleic acids to be followed in real-time. This procedure, called melting curve analysis, uses either dsDNA-specific dyes (e.g., SYBR Green I) or sequence-specific oligonucleotide probes (e.g., HybProbe or SimpleProbe probes), and can be added at the end of PCR.



For a more detailed and up-to-date description of melting curve analysis and genotyping methods, visit [www.lightcycler.com](http://www.lightcycler.com).

### Melting Curve Analysis with SYBR Green I

Melting curve analysis with SYBR Green I is used for product characterization, *i.e.*, to determine whether the desired PCR product is free of nonspecific by-products. PCR products can be characterized by melting curve analysis because each double-stranded DNA molecule has a characteristic melting temperature ( $T_m$ ), at which 50% of the DNA is double-stranded and 50% is melted, *i.e.*, single-stranded. During a melting curve run, the reaction mixture is slowly heated to 95°C, which causes dsDNA to melt. A sharp decrease in SYBR Green I fluorescence occurs when the temperature reaches the  $T_m$  of a PCR product present in the reaction. The LightCycler® Instruments continuously monitor fluorescence during temperature transitions. The software displays these data as a melting curve graph (fluorescence [F] vs. temperature [T]) (Figure 7.4.1, upper graph).



**Figure 7.4.1. Graphs of melting curves and melting peaks from a  $T_m$  Calling analysis of a SYBR Green I experiment.** This example shows how melting temperature analysis can be used for DNA product characterization. The green sample shows only the peak that arises from the desired PCR product, which has a  $T_m$  of 86°C. The blue sample shows this same 86°C peak, but it also shows an additional weak, broad peak with a  $T_m$  of 77°C, which arises from a nonspecific by-product (e.g., a primer-dimer).

# 7

The  $T_m$  of a PCR product present in the reaction can be estimated from the inflection point of the melting curve. However, to make the  $T_m$  easier to see, the software plots a derivative melting curve ( $-dF/dT$ ) in which the center of a melting peak corresponds to the point of inflection. If the PCR generated only one amplicon, melting curve analysis will show only one melting peak. If primer-dimers or other non-specific products are present, they will be shown as additional melting peaks (Figure 7.4.1, lower graph). Checking the  $T_m$  of a PCR product can thus be compared to analyzing the length of a PCR product by gel electrophoresis.

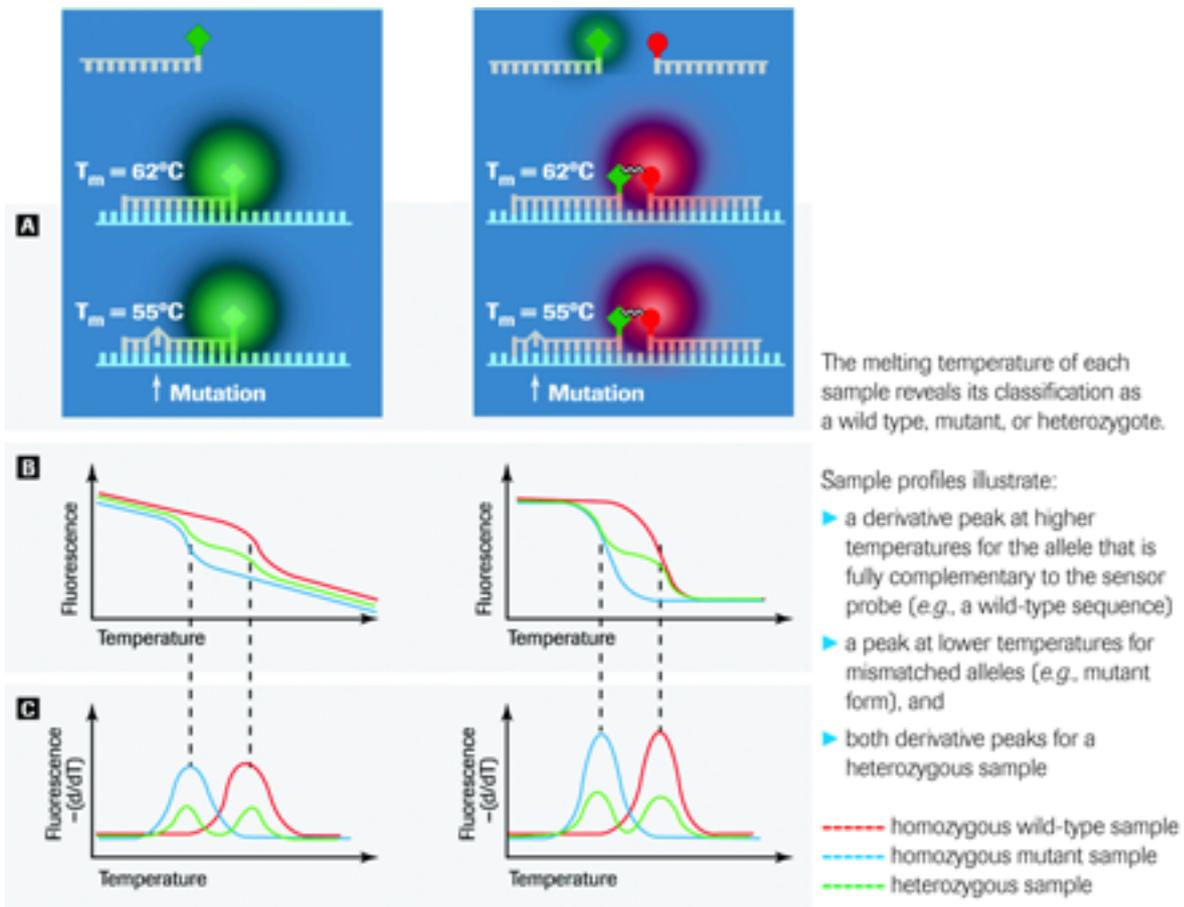
### SNP Detection and Genotyping with HybProbe and SimpleProbe Probes

The precise temperature control of the LightCycler® Carousel-Based System and LightCycler® 480 System allow these instruments to monitor specific fluorescent-labeled probes as they melt off a target sequence. When melting curve analysis is used to monitor the melting of short duplexes, such as hybrids between HybProbe or SimpleProbe probes and target, the assay can identify even single base alterations in the amplicon. Thus, this is an ideal tool for detection of Single Nucleotide Polymorphisms (SNPs) or genotyping.



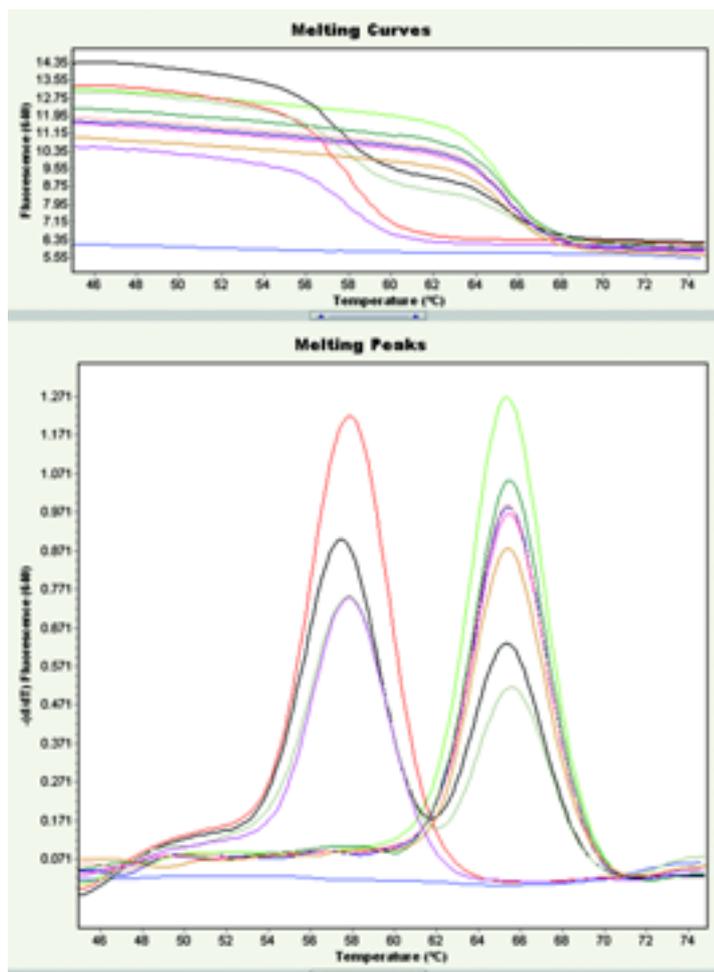
*Although they cannot be used for melting curve analysis, hydrolysis probes can still be used for SNP detection via a different analytical technique. In brief, the technique involves allele-specific hydrolysis probes labeled with different fluorophores. Additional analysis software, which will allow genotyping with hydrolysis probes on the LightCycler® 480 System, will be available at the beginning of 2007. For up-to-date information on genotyping methods, visit [www.lightcycler.com](http://www.lightcycler.com).*

In a genotyping experiment with HybProbe probes, one HybProbe oligonucleotide hybridizes to a part of the target sequence that is not mutated; this probe functions as an anchor probe (Figure 7.4.2). The other HybProbe oligonucleotide (mutation or detection probe) spans the mutation site and has a  $T_m$  approx. 5°C lower than the  $T_m$  of the anchor probe. Immediately after DNA amplification is complete, the LightCycler® Instrument performs a melting curve analysis to distinguish between different genotypes. During this analysis, the temperature is slowly raised (0.1 – 0.2°C/s) and fluorescence is measured continuously. This allows the instrument to monitor the melting behavior of the HybProbe probes that are annealed to the amplicon. As the temperature increases, the shorter mutation probe dissociates first. When this happens, the two dyes are no longer close and the fluorescence signal decreases. Because the  $T_m$  of a probe-target hybrid depends not only on the length and the GC content of the probe, but also on the degree of homology in the hybrid, perfectly bound probes separate at a higher  $T_m$  than those that are bound to DNA containing a destabilizing mismatch (Figure 7.4.3).



**Figure 7.4.2. Schematic representation of mutation detection with SimpleProbe (left) or HybProbe probes (right).** A: Destabilizing mismatches cause a significant reduction of melting temperature ( $T_m$ ); B: melting curve; C: derivative melting peaks.





**Figure 7.4.3. Example of a HybProbe assay for genotyping of a C/T polymorphism.** The upper graph shows sample fluorescence versus temperature. A clear drop in the fluorescent signal can be seen for each sample as the temperature increases. The lower graph plots the first negative derivative of the sample fluorescence versus temperature, and shows the melting temperature of each sample as a peak. Each type of peak represents a different allele: right peak at 66°C = wild type sequence (C); left peak at 56°C = mutant sequence (T). Samples showing both peaks simultaneously are heterozygous for the two alleles.

SimpleProbe assays differ from HybProbe assays in two important ways:

- ▶ Instead of two probes working together, a SimpleProbe assay requires only a single probe (Figure 7.4.2). This probe is designed to specifically hybridize to a target sequence that contains an SNP. Once hybridized to its target sequence, the SimpleProbe probe emits more fluorescence than it does when it is not hybridized. As a result, changes in fluorescence are based solely on the hybridization status of the probe.
- ▶ The SimpleProbe detection format is not based on the FRET principle. If a SimpleProbe probe is free in solution, emission of the reporter dye is quenched by a specific, non-fluorescent quencher. When the probe hybridizes to its target, quenching is reduced and the reporter dye emits fluorescence. A SimpleProbe probe can be labeled at either terminal (the 3′- or 5′-end) or internally (e.g., with the SimpleProbe 519 Labeling Reagent).

For SNP analysis, the melting behavior of the SimpleProbe in the presence of different sequences is determined (Figure 7.4.2). The more stable the hybridization between the SimpleProbe probe and target sequence, the higher the melting temperature. Mutations, such as SNPs, weaken the stability of SimpleProbe probe binding.

## 7.5 Real-Time PCR Instruments Available from Roche Applied Science

### LightCycler® Carousel-Based System: A Proven Standard of Excellence

The LightCycler® Carousel-Based System – including both the LightCycler® 1.5 Instrument and LightCycler® 2.0 Instrument - has repeatedly set the standard for real-time PCR. It was the first system to introduce hybridization probes, true melting curve analysis, automated absolute quantification, and relative quantification with efficiency correction.

Innovative features of the LightCycler® Carousel-Based System include:

- ▶ PCR occurs in specially designed glass capillaries (Figure 7.5.1), which have an optimal surface-to-volume ratio to ensure rapid equilibration between the air and the reaction components. This allows very rapid PCR; a typical amplification cycle requires only 30 to 60 seconds. Up to 32 capillaries can be analyzed in a single run.
- ▶ To avoid contamination, the capillary is tightly closed and does not need to be opened at any time during analysis.
- ▶ A single microvolume fluorimeter is used to monitor fluorescent light generated during PCR and melting curve analysis. For fluorescence excitation, the LightCycler® 2.0 and 1.5 Instruments use a blue LED, which emits light at 470 nm. This LED, which is an integral part of the fluorimeter, needs no maintenance and has a long life span. Fluorescent light is detected by photohybrids.



Figure 7.5.1. LightCycler® capillaries.

This system is available in two different instruments (Figure 7.5.2) to meet your research needs:



- ▶ LightCycler® 1.5 Instrument, which analyzes samples in 20 µl glass capillaries and has three fluorescence detection channels (that measure fluorescence at 530, 640 and 705 nm). Choose the LightCycler® 1.5 Instrument if your work requires only single dye or dual-color assays.



- ▶ LightCycler® 2.0 Instrument, which analyzes samples in either 20 µl or 100 µl glass capillaries and has six fluorescence detection channels (that measure fluorescence at 530, 560, 610, 640, 670 and 705 nm). Choose the LightCycler® 2.0 Instrument if you want to be able to perform multiplex PCR (analysis of up to four different targets simultaneously) in a real-time system.

Figure 7.5.2. LightCycler® 1.5 (upper) and 2.0 (lower) Instruments.

# 7

Both instruments:

- ▶ Are very versatile, since they can be used for both quantitative PCR and SNP analysis. Among the many possible real-time PCR applications are gene expression analysis, microarray result validation, food pathogen testing, and screening for genetically modified organisms.
- ▶ Are very rapid; assay and analysis of samples in 20 µl capillaries can be completed in less than 40 minutes.
- ▶ Are very sensitive; due to the exceptional signal-to-noise ratio of the instruments, a single-copy gene can be detected in one genome equivalent of DNA.
- ▶ Produce very accurate and highly reproducible results (e.g., samples have a coefficient of variation of 0.3% or lower within a run).
- ▶ Can analyze a broad dynamic range of target concentrations (e.g., from 10 to 10<sup>10</sup> copies, depending on the system) in a single run.

### Software for the LightCycler® Carousel-Based System

The LightCycler® Software controls programming and running of the LightCycler® Instruments; it can also perform sophisticated data analysis. LightCycler® Software 3.5.3 is part of the LightCycler® 1.5 Instrument package, while LightCycler® Software 4.x is part of the LightCycler® 2.0 Instrument package.



*Optionally, the LightCycler® 1.5 Instrument can also be used in combination with LightCycler® Software 4.x.*

LightCycler® Software 4.x provides advanced data analysis modules, efficient data management, and effective data protection. This sophisticated software lets you automate your routine analysis, yet highly customize your most demanding analysis. You can streamline your analysis by using one of the enhanced analysis modules:

- ▶ Qualitative Detection, to determine whether a target sequence is present in unknown samples.
- ▶ Absolute and Relative Quantification, to calculate the concentration of your target DNA or RNA in unknown samples based on the concentration of standard samples or a comparison of the ratio of two nucleic acid sequences.
- ▶ Melting Curve Analysis, to analyze sample melting temperatures and profiles to obtain sequence information from the amplified product.
- ▶ Nucleic Acid Quantification, to determine the concentration of nucleic acids without running an amplification reaction.



*For a more detailed and up-to-date description of the LightCycler® 1.5 and 2.0 Instruments, visit [www.lightcycler.com](http://www.lightcycler.com).*

### LightCycler® 480 Real-Time PCR System: Cutting-Edge, Real-Time PCR Technology for Medium- and High-Throughput Applications

The LightCycler® 480 Real-Time PCR System (Figure 7.5.3) extends LightCycler® System accuracy, speed, and versatility to medium- and high-throughput applications in gene expression and genotyping analysis.



Figure 7.5.3. LightCycler® 480 Real-Time PCR System.

Several features of this system make it readily adaptable to such applications:

- ▶ The LightCycler® 480 Instrument is a compact and versatile benchtop instrument that can hold multiwell plates for either 96 or 384 samples. The two available thermal block cyclers (for 96 or 384 samples) can easily be interchanged, making the instrument readily adaptable to different throughput needs. Calibration is not required after block exchange.
- ▶ The LightCycler® 480 System uses special multiwell plates that fit optimally in the LightCycler® 480 thermal block cycler and are an integral part of the system. Thus, the instrument is capable of running PCRs on a very broad range of sample volumes, ranging from 5 to 20  $\mu\text{l}$  (for a 384-well plate) or 20 to 100  $\mu\text{l}$  (for a 96-well plate). This allows assay design to be highly flexible (*e.g.*, when sensitivity is crucial because of low target concentrations).
- ▶ The LightCycler® 480 System uses a novel type of thermal block cycler, which incorporates Thermo-Base technology to ensure optimal heat transfer and distribution to all samples on a multiwell plate. This efficient heat transfer leads to excellent well-to-well temperature homogeneity and maximal inter-well, inter-cycle reproducibility. This technology enables a 40-cycle PCR run to be completed in less than 40 minutes (in the 384-well format).
- ▶ For fluorescence excitation, the LightCycler® 480 System uses a high-intensity xenon lamp. The broad spectrum of this light source supports the use of a wide range of dyes and at the same time provides maximal sensitivity for a broad range of assay formats. To make excitation as specific as possible and to reduce crosstalk between channels when more than one dye is used, the LightCycler® 480 System includes five different excitation filters (wavelength maxima: 450, 483, 523, 558 and 615 nm) and six different emission filters (wavelength maxima: 500, 533, 568, 610, 640, 670 nm) (Table 7.5.1). These filters can be freely combined to accommodate the requirements of the probe format and dyes used in an experiment (Table 7.5.2).



**Table 7.5.1: Assay Formats Supported by the LightCycler® 480 System.**

<b>Xenon lamp</b>							
<b>Excitation filters</b>	450	483	483	523	558		615
<b>Emission filters</b>	500	533	533	568	500	640	670
<b>Dyes (e.g.)</b>	LightCycler® Cyan 500	SYBR Green I	Fluorescein (Fluos / FAM)	HEX (VIC)	LightCycler® Red 610	LightCycler® Red 640	Cy5
<b>Detection formats</b>	Hydrolysis Probe (R)	SYBR Green I	Hydrolysis probe (R), HybProbe probe (D), SimpleProbe probes (R)	Hydrolysis probe (R), HybProbe probe (A)			

Legend: Reporter (R), Donor (D), Acceptor (A).

**Table 7.5.2: Detection Formats, Dyes and Applications Supported by the LightCycler® 480 System.**

Excitation (nm)	Detection (nm)	Assay format	Dyes	Applications
483	533	SYBR Green I	SYBR Green I	Characterization Quantification
483	610	HybProbe probes	LightCycler® Red 610	Quantification
483	640		LightCycler® Red 640	SNP analysis
483	670		Cys	
450	500	Hydrolysis probes	LightCycler® Cyan 500	Quantification
483	533		FAM	SNP analysis
523	568		VIC / HEX	
558	610		LightCycler® Red 610	
558	640		LightCycler® Red 640	
615	670		Cys	
483	533	SimpleProbe probes	Fluorescein	SNP analysis

- ▶ For signal capture, a large field lens and a charge-coupled device (CCD) camera are arranged to ensure uniform collection of signals across the plate. Fluorescence detection is therefore largely independent of sample position, thereby eliminating the need for additional reference dyes (e.g., ROX) to compensate for two-dimensional differences in intensity.
- ▶ The LightCycler® 480 System includes a basic software module that allows users to easily set up reaction protocols and run absolute quantification or melting curve analysis. The software is modular and can be expanded with additional software modules (e.g., for relative quantification or genotyping).
- ▶ For integrated workflow monitoring, the LightCycler® 480 System offers connectivity to standard LIMS (laboratory information management systems) and compatibility with CFR 21 Part 11 standards.



For a more detailed and up-to-date description of the LightCycler® 480 System, visit [www.lightcycler.com](http://www.lightcycler.com).

## 7.6 Real-Time PCR Reagents

Experiments that analyze expression of low-abundance messages or detect SNPs and other mutations require highly specific and efficient reaction conditions, optimal primer design, and absence of contaminants. Such high specificity PCRs avoid the accumulation of side-products or artifacts, and prevent primer-dimer formation.

The LightCycler® Systems help ensure high specificity PCRs by offering a unique combination of rapid cycling and convenient master mixes. This section gives a brief overview of those real-time PCR reagent mixes.

### PCR Reagents for the LightCycler® Carousel-Based System

Roche Applied Science offers several convenient master mixes that:

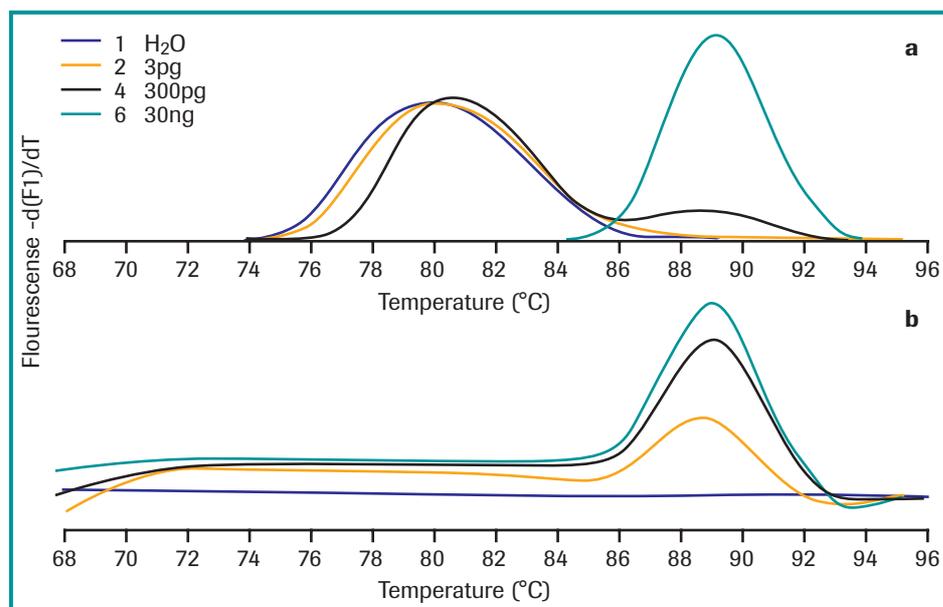
- ▶ Contain FastStart Taq DNA Polymerase, the optimal enzyme for hot-start reactions
- ▶ Contain a MgCl<sub>2</sub> concentration that is optimal for a specific application
- ▶ Save time because they are ready-to-use, concentrated, and substitute for several reagents that would otherwise have to be pipetted separately



See Chapter 4 for details on hot-start PCR and the FastStart Taq DNA Polymerase in general.

### LightCycler® FastStart DNA Master

The LightCycler® FastStart DNA Master is a ready to use hot-start reaction mix (10× conc.). This master is available for either the HybProbe or SYBR Green detection format. The FastStart Taq DNA Polymerase in the mix has been optimized for use in the LightCycler® Capillaries, to ensure rapid and specific DNA amplification (Figure 7.6.1). The LightCycler® FastStart DNA Masters are ideally suited for quantification, SNP and mutation detection, and two-step RT-PCR on the LightCycler® Carousel-Based System Instruments.

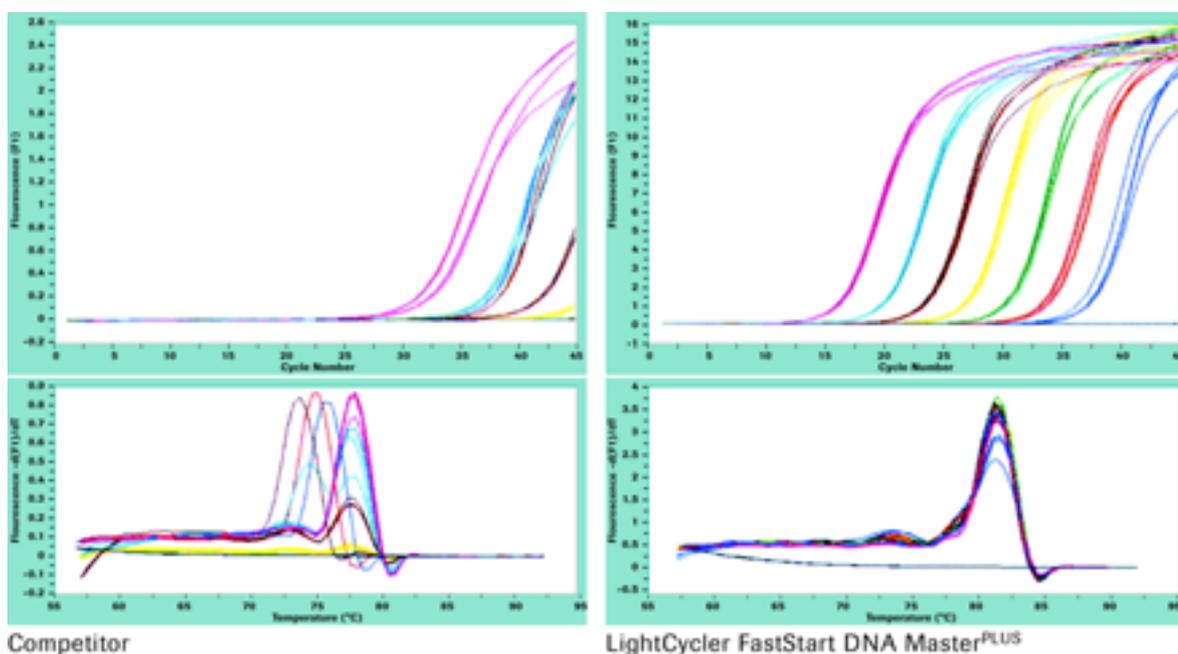


**Figure 7.6.1. Efficiency of hot start reactions with the LightCycler® FastStart DNA Master SYBR Green I.** A 316 bp fragment has been amplified from 30 ng, 300 pg, and 3 pg of human genomic DNA with specially selected primers that tend to cause formation of primer-dimers. Identical protocols and primers were used to generate melting curves with the LightCycler® DNA Master SYBR Green I (panel a) and the LightCycler® FastStart DNA Master SYBR Green I (panel b). A negative control, in which PCR Grade water substituted for template, was also included in the runs. LightCycler® FastStart DNA Master SYBR Green I clearly reduces the amount of primer-dimers formed and therefore increases the sensitivity of the reaction.

### LightCycler® FastStart DNA Master<sup>PLUS</sup>

The LightCycler® FastStart DNA Master<sup>PLUS</sup> (available for either HybProbe or SYBR Green I detection) offers a new dimension of convenience and performance for hot-start PCR because:

- ▶ The reaction buffer and enzyme are optimized for robustness and make the reaction less susceptible to PCR inhibitors.
- ▶ The MgCl<sub>2</sub> concentration, tested on a large variety of targets, has already been optimized in the reaction buffer, thus eliminating tedious MgCl<sub>2</sub> titration.
- ▶ The mix delivers more PCR product and higher specificity than other hot-start reaction mixes (Figure 7.6.2).



**Figure 7.6.2. Performance of a dilution series** (from 10<sup>6</sup> to 1 copy/μl) in a real-time PCR assay with either a PCR Master from a competitor or the LightCycler® FastStart DNA Master<sup>PLUS</sup>.

The LightCycler® FastStart DNA Master<sup>PLUS</sup> master mixes (5× conc.) are ideally suited for applications that require high sensitivity and reliability. They are designed for quantitative PCR on the LightCycler® Carousel-Based System, but are also suitable for genotyping via melting curve analysis. Furthermore, they can be used to perform two-step RT-PCR.

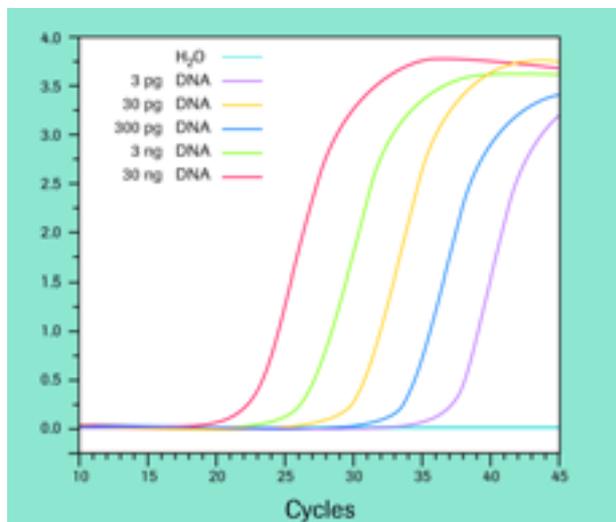


*LightCycler® FastStart DNA Master<sup>PLUS</sup> is also available for 100 μl reactions in the 100 μl LightCycler® Capillaries. Even though a 20 μl reaction volume provides sufficient sensitivity for most applications, a 100 μl reaction volume can increase the probability of detecting extremely low abundance targets.*

### LightCycler® TaqMan® Master

The LightCycler® TaqMan® Master allows you to run hydrolysis probe-based assays on the LightCycler® 2.0 and 1.5 Instruments without having to adapt the assay or do elaborate testing and optimization. This 5× master mix is designed to take advantage of the features of the LightCycler® Carousel-Based System's optical unit, thus ensuring high sensitivity and specificity in mono- or dual-color real-time PCR assays. This master mix allows you to:

- ▶ Use hydrolysis probes to perform sensitive and quantitative PCR or RT-PCR on the LightCycler® Carousel-based System (Figure 7.6.3).
- ▶ Eliminate time-consuming MgCl<sub>2</sub> titration.
- ▶ Achieve consistent, high-quality performance with the LightCycler® 2.0 and 1.5 Instruments.

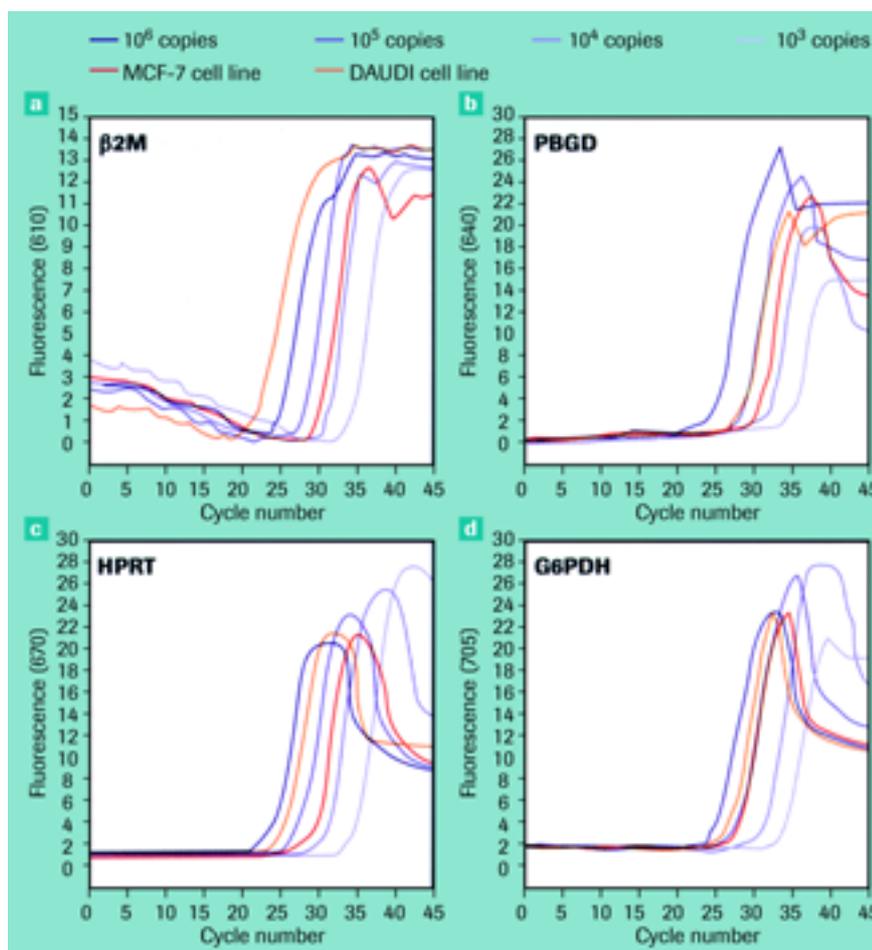


**Figure 7.6.3. Amplification and detection of the human cyclophilin A gene on the LightCycler® 2.0 Instrument**, using dilutions of genomic DNA as templates, a set of gene-specific primers and a FAM/TAMRA-labeled hydrolysis probe.

7

### LightCycler® Multiplex Master HybProbe

With its six fluorescence detection channels, the LightCycler® 2.0 Instrument is ideally suited for multiplex reactions that detect up to four different targets (Figure 7.6.4). Multiplex reactions demand a higher degree of specificity and sensitivity than mono- or dual-color reactions. Competing parameters, different target concentrations, and the need for very specific primer/probe pairs make the use of an effective buffer/enzyme system imperative for reliable amplification and sensitivity. This is why the LightCycler® Multiplex DNA Master HybProbe contains a special form of Taq DNA Polymerase (5'-exonuclease-minus, N-terminal deleted form of recombinant Taq DNA Polymerase) that is specifically optimized for higher processivity and specificity. Buffer conditions, including optimal enzyme and MgCl<sub>2</sub> concentrations, were specifically designed and evaluated for multiplex reactions.



**Figure 7.6.4. cDNA amplification assays of four housekeeping genes** [ $\beta_2$  microglobulin ( $\beta_2$ M), porphobilinogen deaminase (PBGD), hypoxanthine phosphoribosyl transferase (HPRT), and glucose-6-phosphate dehydrogenase (G6PDH)] from two different human cell lines, DAUDI and MCF-7. The MgCl<sub>2</sub> concentration of the master mix was not adjusted. For each sample, the amount of the four different RNAs was analyzed in a single capillary with a multicolor detection system.

As a positive control, a reference control RNA from the LightCycler® h-Housekeeping Gene Selection Set was diluted from 10<sup>6</sup> to 10<sup>3</sup> copies/ $\mu$ l for each target. For each concentration, the controls were amplified in a single reaction.

The results show that the LightCycler® Multiplex DNA Master HybProbe allows measurement of four parameters with varying expression profiles in a single reaction.



All real-time PCR kits available for the LightCycler® Carousel-Based System contain a dNTP mix that contains dUTP instead of dTTP. Thus, they can be used with heat-labile Uracil-DNA Glycosylase to prevent carryover during PCR.

## RT-PCR Reagents for the LightCycler® Carousel-Based System

Two approaches can be used for RNA quantification with the LightCycler® Carousel-based System: either one-step RT-PCR or two-step RT-PCR. Each approach has its advantages, depending on the experimental set-up. Dedicated master mixes are offered for each type of RT-PCR.

### Master Mixes for One-step RT-PCR

The main advantage of one-step RT-PCR is that the reaction requires fewer steps to set up, thus minimizing loss of sample and risk of contamination.

The LightCycler® RNA Masters (available for either HybProbe or SYBR Green I detection) are ready-to-use hot-start reaction mixes that contain Tth DNA Polymerase and Aptamers.

Tth DNA Polymerase, a thermostable enzyme with RNA-dependent reverse transcriptase activity and DNA-dependent polymerase activity, is the ideal enzyme for performing consecutive RT and PCR in a single tube without compromising one reaction for the other (Figure 7.6.5). The high reaction temperature of this polymerase allows efficient transcription of sequences with high GC content or secondary structures.

Aptamers are oligonucleotides that bind to the active center of the Tth DNA Polymerase and prevent the enzyme from attaching to nucleic acid targets at temperatures below its optimal reaction temperature. They are released from the enzyme at higher temperatures, thus allowing a hot start reaction.

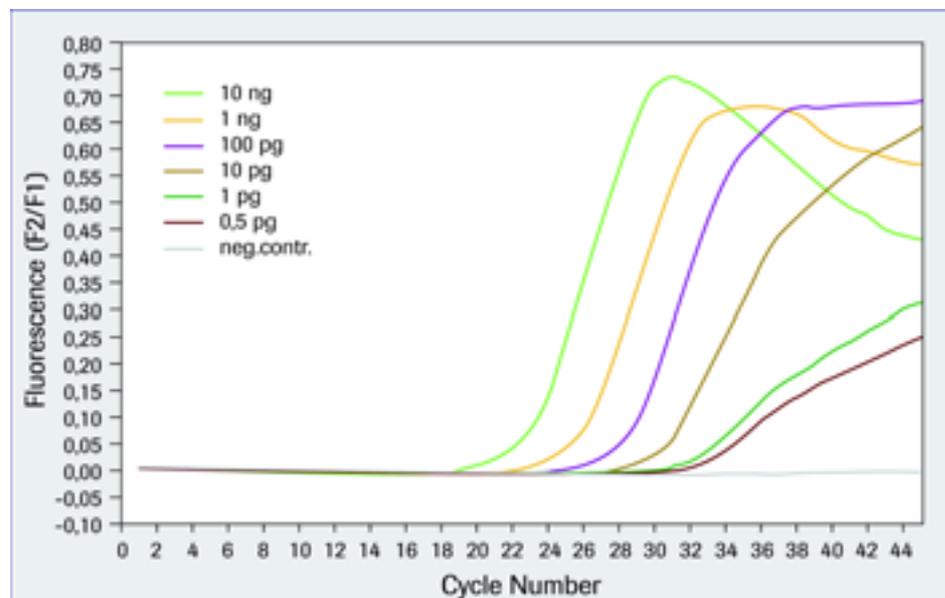


Figure 7.6.5. Detection of GAP3PDH in serial dilutions of total liver RNA with the LightCycler® RNA Master HybProbe.

### Enzymes, Kits and Master Mixes for Two-Step RT-PCR

When high yields of full-length cDNA are required, two-step RT-PCR is the preferred approach and Transcriptor Reverse Transcriptase is the preferred enzyme for the RT step.

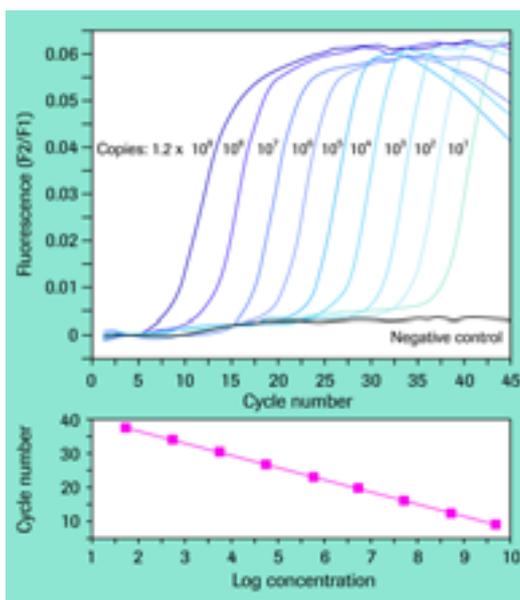
The new recombinant enzyme, Transcriptor Reverse Transcriptase, has an unwinding activity and an RNase H activity that degrades RNA in RNA:DNA hybrids. This eliminates the need for an additional time-consuming RNase H step after reverse transcription. Transcriptor Reverse Transcriptase provides unbiased, high-fidelity transcription of RNA, even when it contains problematic sequences (GC-rich, high secondary structure). This makes the enzyme ideal for generating high yields of full-length cDNA, and makes it ideally suited for RT-PCR assays on the LightCycler® Carousel-Based System (Figures 7.6.6 and 7.6.7).

Transcriptor Reverse Transcriptase is available as either a stand-alone enzyme or as part of the First Strand cDNA Synthesis Kit. This kit contains all reagents required for the RT step, including a random hexamer primer and an anchored-oligo(dT)<sub>18</sub> primer.



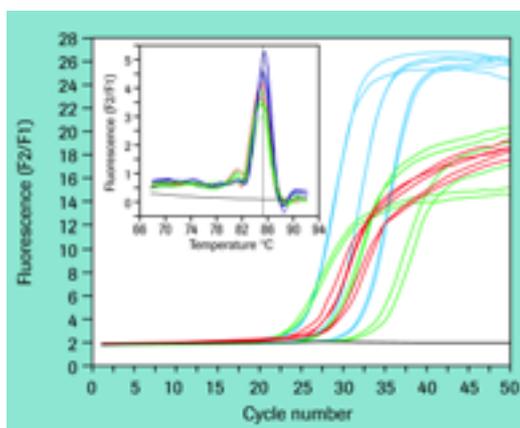
For details on Transcriptor Reverse Transcriptase and the Transcriptor First Strand cDNA Synthesis Kit, see Chapter 5.

All LightCycler® Carousel-Based System PCR reagents are compatible to cDNA samples generated with Transcriptor Reverse Transcriptase.



**Figure 7.6.6. Two-step RT-PCR, using Transcriptor Reverse Transcriptase for the RT step.** Various amounts (5 to  $5 \times 10^9$  copies) of *in vitro* transcribed porphobilinogen deaminase (PBGD) RNA were reverse transcribed with Transcriptor Reverse Transcriptase in a total volume of 20  $\mu$ l. Five  $\mu$ l of each cDNA was amplified in a subsequent PCR with the LightCycler® FastStart DNA Master HybProbe, along with primers and HybProbe probes specific for PBGD.

**Result:** The assay was linear and showed a broad dynamic range of products (from 12 to  $1.2 \times 10^9$  copies PBGD per capillary), indicating adequate transcription and amplification of a  $10^8$ -fold range of input RNA.



**Figure 7.6.7. Comparison of Transcriptor RTase with other RTases.** Various amounts (10 to 1,000 ng) of human skeletal muscle total RNA were reverse transcribed with three different reverse transcriptases, using oligo(dT) priming. The subsequent PCR was performed with primers specific for the human dystrophin gene. A 372-bp fragment was amplified with LightCycler® FastStart DNA Master SYBR Green. The  $Mg^{2+}$  concentration was optimized for each PCR. All reactions were run in duplicate.

**Result:** Transcriptor Reverse Transcriptase produces curves with higher fluorescence intensity and lower crossing points than other reverse transcriptases.

## PCR Reagents for the LightCycler® 480 System

The LightCycler® 480 System includes master mixes (Table 7.6.1) that are tailor-made for each of the main LightCycler® 480 applications (gene identification, gene quantification, genotyping). These master mixes are all stable at room temperature for extended periods of time, ensuring maximum robustness in automated high-throughput applications. Enzymes and buffer conditions have been carefully chosen and optimized for each application:

- ▶ LightCycler 480® SYBR Green I Master contains components to help minimize primer-dimers.
- ▶ LightCycler 480® Probes Master is optimized for quantitative PCR assays with hydrolysis probes.
- ▶ LightCycler 480® Genotyping Master is optimized for melting curve analysis with HybProbe or SimpleProbe probes.

All LightCycler® 480 PCR master mixes are ready-to-use, single-solution reagents. Setting up a reaction requires only addition of template DNA, primers and (except for experiments with SYBR Green I) probes. The mixes can be used with different types of DNA (e.g., genomic DNA, cDNA) and are ideally suited for high-throughput applications in 96- or 384-well plates. The MgCl<sub>2</sub> concentration in each master mix is optimized and works with nearly all primer combinations. No adjustment of the MgCl<sub>2</sub> concentration is needed to amplify different sequences. LightCycler® 480 PCR Master Mixes are all based on enzymes compatible with hot-start protocols. LightCycler® 480 Master Mixes can be used in two-step RT-PCR applications (e.g., with Transcriptor Reverse Transcriptase).



*The LightCycler® 480 PCR Master Mixes have been adapted for the rapid cycling environment of the LightCycler® 480 System and for the different probe chemistries supported by the system. Optimal experimental results can therefore only be obtained when these reagents are used in the LightCycler® 480 System. Due to the differences in design (dimensions, material) of the instruments, reagents that are tailor-made for one LightCycler® System (LightCycler® 480 System or the LightCycler® Carousel-Based System with capillaries) should not be used on instruments from the other system.*



See Table 7.6.1 for an overview of available LightCycler® 480 System PCR reagents:

**Table 7.6.1: Master Reagent Mixes for the LightCycler® 480 System.**

Product Name	Main Application	Enzyme	Probe Formats Supported
LightCycler® 480 SYBR Green I Master (2× conc.)	Qualitative gene detection and absolute quantification	FastStart Taq DNA Polymerase	SYBR Green I
LightCycler® 480 Probes Master (2× conc.)	Absolute and relative gene quantification	FastStart Taq DNA Polymerase	<ul style="list-style-type: none"> <li>▶ Optimized for hydrolysis probes (e.g., Universal ProbeLibrary probes)</li> <li>▶ Can be adapted to other probe formats (e.g., HybProbe probes, Molecular Beacon, and Scorpions)</li> </ul>
LightCycler® 480 Genotyping Master (5× conc.)	<ul style="list-style-type: none"> <li>▶ Genotyping via melting curves</li> <li>▶ SNP genotyping and mutation analysis</li> <li>▶ Recommended for multiplex assays</li> </ul>	Thermostable recombinant Taq DNA Polymerase that is 5'-exonuclease-minus and has an N-terminal deletion	<ul style="list-style-type: none"> <li>▶ HybProbe probes</li> <li>▶ SimpleProbe probes</li> <li>! This mix should NOT be used with hydrolysis probes.</li> </ul>



To perform RT-PCR reactions on the LightCycler® 480 System, combine the LightCycler® 480 System PCR reagents with the Transcriptor First Strand cDNA Synthesis Kit (or the stand-alone enzyme Transcriptor Reverse Transcriptase). All LightCycler® 480 System PCR reagents are fully compatible to cDNA samples generated with Transcriptor Reverse Transcriptase. One-step RT-PCR reagents for the LightCycler® 480 System will be available from the second half of 2006 on.

### PCR Reagents for Real-Time PCR Systems (other than the LightCycler® Systems)

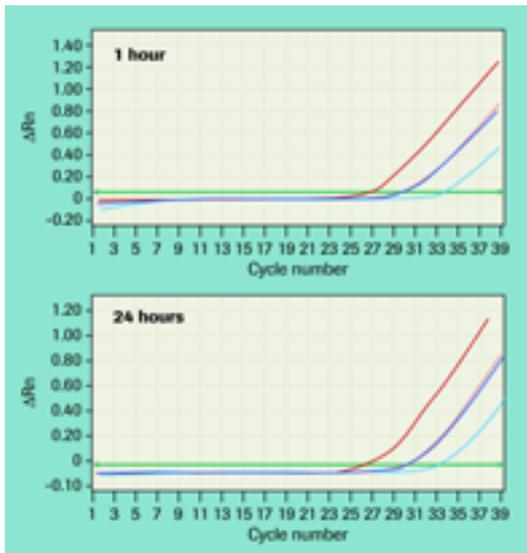
The FastStart TaqMan® Probe Master and the FastStart SYBR Green Master are ready-to-use reagents mixes that simplify preparation of samples for quantitative PCR (qPCR) and RT-PCR (qRT-PCR). These master mixes are optimized for use with most real-time PCR instruments (other than the LightCycler® Instruments). They are each available in two formulations, one that contains the ROX Reference Dye and one without ROX.

These 2× concentrated master mixes:

- ▶ Contain all reagents (except primers, template and probe) needed for running quantitative, real-time PCR assays.
- ▶ Are suitable for amplification and detection of any DNA or cDNA target up to 500 bp length, even if they are GC-rich.
- ▶ Do not require addition of MgCl<sub>2</sub>, thereby eliminating time-consuming optimization steps.
- ▶ Contain dUTP instead of dTTP, so they can be used with Uracil-DNA Glycosylase to prevent false-positive signals from carryover contamination.
- ▶ Are stable – even supplemented with template, primers (and probes) – for at least 24 hours at room temperature (Figure 7.6.8).

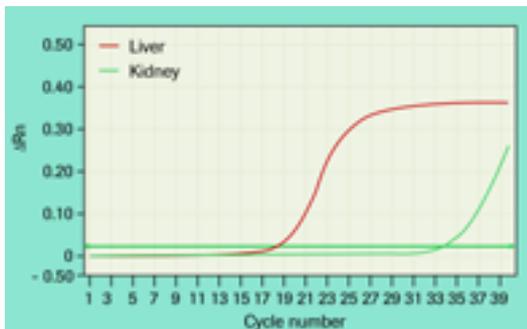


This stability is especially important when reactions are set up robotically, since automated set-up requires the reaction mix to be stable over a specific period of time.



**Figure 7.6.8. Stability of a complete reaction mix made with the FastStart TaqMan<sup>®</sup> Probe Master.** Aliquots of the complete reaction mix were left standing for 1 h or 24 h at room temperature, then analyzed on an Applied Biosystems 7500 Real-Time PCR System.

If you combine either of these master mixes with the Transcriptor First Strand cDNA Synthesis Kit you will have a system that gives superior results in two-step qRT-PCR (Figure 7.6.9). The Transcriptor First Strand cDNA Synthesis Kit is designed and tested for qPCR; it is efficient with all real-time PCR instruments.



**Figure 7.6.9. Quantification of the tubulin gene in different rat tissues with the FastStart SYBR Green Master.** mRNA was reverse transcribed with the Transcriptor First Strand cDNA Synthesis System. The cDNA product was then amplified on an Applied Biosystems 7500 Real-Time PCR System.

A stock solution (1 mM) of ROX Reference Dye in water is also available. It can be used as an additive for quantitative, real-time DNA-detection assays, including qPCR and two-step qRT-PCR. If included in a reaction, this reference dye can compensate for variations in fluorescence between wells. Because the concentration of the dye does not vary during PCR, it produces a constant fluorescent signal. This signal provides a stable baseline, which allows sample normalization.



*Well-to-well variations can be due to the design of the instrument or pipetting errors. However, if you use one of the LightCycler<sup>®</sup> Systems, you do not need to include ROX Reference Dye in the reactions because the LightCycler<sup>®</sup> Instruments are designed to minimize well-to-well variations.*

## 7.7 Published Examples of Applications for the LightCycler® Carousel-Based Systems

The reliability and versatility of the LightCycler® Systems and real-time PCR assay formats have been shown in publication after publication. Here is a small sample of the published papers that used real-time PCR methods and instruments available from Roche Applied Science:

Albanese, E., Yang, X.Y. and Fernandes, H. (2003) *Identification of Cytokine SNPs Using LightCycler® Hybridization Probes and Melting Curve Analysis*. *Biochemica* 02/2003, 4-5.

Aslanidis, C. and Schmitz, G. (2001) *Alpha-1-antitrypsin mutation detection on the LightCycler® using human genomic DNA from blood research samples isolated on the MagNA Pure LC*. *Biochemica* 02/2001, 5-8.

Bay, S, Wittwer, C.T., King, T.C. and Elelitoba- Johnson, K.S.J. (1999) *Fluorescence melting curve-based analysis for the detection of the bcl-1/JH translocation in mantle cell lymphoma*. *Lab. Invest.* 79, 337-345.

Bernard, P.B. and Wittwer, C.T. (2000) *Homogenous amplification and variant detection by fluorescent hybridization probes*. *Clin. Chem.* 46, 147-148.

Bernard, P.S., Ajioka, R.S., Kushner, J.P. and Wittwer, C.T. (1998). *Homogenous multiplex genotyping of hemochromatosis mutations with fluorescent hybridization probes*. *Am. J. Path.* 153,1055-1061.

Bernard, P.S., Lay, M.J. and Wittwer, C.T. (1998) *Integrated amplification and detection of the C677 T point mutation in the methylenetetrahydrofolate reductase gene by fluorescence resonance energy transfer and probe melting curves*. *Anal. Biochem.* 255, 101-107.

Brown, R.A., Lay, M.J. and Wittwer, C.T. (1998) *Rapid cycle amplification for construction of competitive templates*. In: *Genetic Engineering with PCR* (Horton, R.M. & Tait, R.C., eds.), Horizon Scientific Press, Norfolk, England, pp. 57-70.

Farwick, M., Brehme, J., Olbermann, P., Huthmacher, K. and Pfefferl, W. (2002) *Analysis of bacterial transcripts by combined application of DNA arrays and quantitative RT-PCR*. *Biochemica* 01/2002, 4-5.

Flori, A.R., Hader, C. and Schulz, W.A. (2005) *Screening for DNA hypermethylation using the LightCycler® Instrument*. *Biochemica* 01/2005, 4-6.

Gaede, W.. *Detection of classical swine fever with the LightCycler Instrument*. *Biochemica* 03/2002, 4-6.

Guo, Z., Liu, Q. and Smith, L.M. (1997) *Enhanced discrimination of single nucleotide polymorphism by artificial mismatch hybridization*. *Nature Biotech.* 4, 331-335.

Hayward, A.L., Oefner, P.J., Sabatini, .S, Kainer, D.B., Hinojos, C.A. and Doris, P.A. (1998) *Modeling and analysis of competitive RT-PCR*. *Nucleic Acids Res.* 26(11), 2511-2518.

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### **Disclaimer**

For the LightCycler® 1.5 Instrument, Cat. No. 04 484 001, the LightCycler® 2.0 Instrument, Cat. No. 03 532 414 201, see Disclaimer Nos. 7, and 8;

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*Chapter 8*  
**Applications**

8	Applications	Page
	General Introduction .....	229
8.1	Multiplex PCR Using the FastStart High Fidelity PCR System.....	230
8.2	Specific Amplification of Difficult PCR Products from Small Amounts of DNA Using FastStart Taq DNA Polymerase .....	238
8.3	FastStart Taq DNA Polymerase Is Ideally Suited for RT-PCR of Laser Captured Microdissected Material .....	242
8.4	Cloning of mRNAs and Rapid Screening by Direct Colony PCR with the FastStart PCR Master .....	244
8.5	FastStart High Fidelity PCR System Simplifies Study of Epigenetics and DNA Methylation .....	250
8.6	Analysis of DNA Methylation Patterns at the BRCA1 CpG Island.....	254
8.7	Comparison of Several Hot-Start Taq DNA Polymerases for Detection of Differentially Expressed Genes by GeneFishing .....	259
8.8	Transcriptional Organization of the O Antigen Biosynthesis Cluster in the GC-Rich Bacterium Burkholderia cenocepacia .....	262
8.9	Transcriptional Analysis of a Retroviral Vector System that Transfers Intron-Containing Genes .....	265
8.10	Quantification of BRCA1 Expression Levels with Standard Roche RT-PCR Reagents: A Sensitive Method for Detecting Low Amounts of Transcripts.....	271
8.11	Tailor-made Solutions Exemplified with the High-throughput 5' RACE Kit .....	276



## 8. Applications

### General Introduction

This chapter presents a few of the many papers submitted to Roche Applied Science (RAS). Each describes an application for one or more of the PCR and RT-PCR products described in previous chapters. This selection of papers amply illustrates how Roche Applied Science products save precious time in the development of experimental applications that range from multiplex PCR (section 8.1) to direct colony PCR (section 8.4).

The authors of these papers, whether from Roche Applied Science laboratories or from outside laboratories, have one thing in common. They all found that Roche Applied Science products made their experiments easier. For example, Hermann and Foerzler (section 8.2) showed how FastStart Taq DNA Polymerase increased the sensitivity of PCR for difficult-to-amplify PCR products. Paduch (section 8.5) found that the FastStart High Fidelity PCR System made it possible to perform methylation-specific PCR, an important technique for the study of epigenetic mechanisms, without requiring extensive optimization. Ortega and Valvano (section 8.8) reported that the combination of Transcriptor Reverse Transcriptase and Taq DNA Polymerase formed an efficient two-step RT-PCR system for amplification of RNA templates with up to 70% GC content.

In some cases, these products not only made their experiments easier, they made them possible! See for instance the article by Pohjanvirta (section 8.7), who found that FastStart Taq DNA Polymerase was the only hot-start enzyme that amplified all the expected products in his experimental system.



## 8.1 Multiplex PCR Using the FastStart High Fidelity PCR System

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### Introduction

The Roche Applied Science FastStart High Fidelity PCR System is the product of choice for multiplex PCR. The multiplex PCR system can easily be developed and optimized in three simple, straightforward steps:

- ▶ Monoplex PCR of all fragments separately
- ▶ Initial multiplex PCR optimization with buffers from the PCR Optimization Kit
- ▶ Final multiplex PCR optimization with the optimal PCR buffer and different additives

Here, we describe a detailed, step-by-step protocol for developing such a multiplex PCR system. This protocol is suitable for many experimental systems, even when the primer set is very complex. As this article demonstrates, the system can amplify up to 18 PCR fragments simultaneously after the reaction is optimized.

### Materials and Methods

#### Preparation of Template

Suitable DNA could be prepared with any of a variety of Roche Applied Science reagents that are specifically designed for the preparation of high quality nucleic acids. (For details, see Chapter 3 in this manual.)

RNA targets were amplified with a two-step RT-PCR procedure. For the first step, the Transcriptor First Strand cDNA Synthesis Kit and an anchored oligo(dT) primer were used to produce a cDNA template suitable for PCR. (The RT procedure was as described in section 5.4 of Chapter 5 in this manual.) An aliquot of the synthesized cDNA was used directly in each of the monoplex and multiplex reactions described below.

For the entire three step protocol, a total of at least one monoplex reaction for each individual primer pair and 31 multiplex optimization reactions were needed. As template, each of these monoplex and multiplex reactions required either 200 ng of isolated DNA or 5 µl of cDNA (obtained directly from the RT reaction).

#### Selection of Targets and Design of PCR Primers

Specific primer sets for multiplex applications were designed with LightCycler<sup>®</sup> Probe Design Software 2.0 (from Roche Applied Science). For a detailed description of how this software may be used to design primer sets for multiplex PCR, see Schubert *et al.* (2005).

#### Step 1: Monoplex PCR of All Fragments Separately

The procedure below was used to test each primer pair individually under identical PCR and cycle conditions.



- 1 Set up each monoplex PCR at room temperature by adding the components below:

### Components of Each Monoplex Reaction

Components (Mix 1)	Volume <sup>a</sup>	Final conc.
10× FastStart High Fidelity Reaction Buffer with MgCl <sub>2</sub> *	5 µl	1× (includes 1.8 mM MgCl <sub>2</sub> )
PCR Grade Nucleotide Mix, 10 mM each*	1 µl	200 µM each
DNA or cDNA Template	variable	200 ng DNA or 5 µl cDNA (from a 20 µl reaction) <sup>b</sup>
Upstream Primer	variable	0.4 µM
Downstream Primer	variable	0.4 µM
FastStart High Fidelity Enzyme Blend*	0.5 µl	2.5 U
PCR Grade Water*	to make vol = 50 µl	
<b>Total volume</b>	<b>50 µl</b>	

\* Available from Roche Applied Science.

<sup>a</sup>) For simplification, prepare a Master Mix of all components except the primers, aliquot into separate tubes and add the appropriate primers to each tube separately.

<sup>b</sup>) The tested cDNAs were produced with the Transcriptor First Strand cDNA Synthesis Kit (Roche Applied Science). Five µl of the product mixture is required for each monoplex PCR.

- 2 Mix the reagents in each tube and centrifuge briefly.
- 3 Place PCR tubes in the PCR instrument and begin thermal cycling according to the profile below.
- 4 Separate the products (3–5 µl monoplex reaction/lane) for 1 hour at 100 V on a 1 – 4% Agarose MP (Roche Applied Science) gel containing 0.1% ethidium bromide.  
 Use 1% agarose to separate products >1000 bp. Use 3 – 4% agarose to separate products ranging from 50 bp to 1000 bp.
- 5 If necessary, adjust the thermal cycling program until a single program works for all the monoplex PCRs.  
 Each of the monoplex PCRs should generate specific single products.

 The thermal profile below was developed for the Applied Biosystems GeneAmp PCR System 2400 thermal block cycler. Other PCR instruments may require a different profile.

Cycles	Temperature	Time
<b>Activation</b>		
1	95°C	4 minutes <sup>a</sup>
<b>Amplification</b>		
30 <sup>b</sup>	Denaturation	95°C
	Annealing	60°C <sup>c</sup>
	Elongation	72°C
		1 minute
		1 minute
		1–2 minutes <sup>d</sup>
<b>Final Elongation</b>		
1	72°C	7 minutes

<sup>a</sup>) Activate the FastStart High Fidelity Enzyme Blend for at least 4 min. Increase activation time (up to 10 min) for difficult amplifications or to increase the yield of product.

<sup>b</sup>) Increase the number of cycles to increase the yield of product.

<sup>c</sup>) Annealing temperature depends on the melting temperature of the primers.

<sup>d</sup>) We recommend using 1 min for 1.0 kb targets, 2 min for targets up to 1.8 kb.



## Step 2: Initial Optimization of Multiplex PCR with Buffers from the PCR Optimization Kit

After detectable amounts of all monoplex amplification products had been generated under the same PCR conditions, these conditions had to be optimized for multiplex PCR. During the initial optimization described below, the PCR Optimization Kit (Roche Applied Science) was used to define the optimal multiplex PCR buffer. This step required a total of 17 multiplex reactions.

- At room temperature, set up a master mix by adding the components below:  
 *The extra volume in the master mix is to compensate for pipetting losses in step 2 below.*

### Master Mix for All Reactions

Components (Mix 1)	Volume	Final amount (for each reaction)
PCR Grade Nucleotide Mix, 10 mM each	18 µl	200 µM each
DNA or cDNA Template	variable vol. of DNA or 90 µl cDNA	200 ng DNA/reaction or 5 µl cDNA/reaction <sup>a</sup>
Multiplex Primer Mixture	variable	0.4 µM each
FastStart High Fidelity Enzyme Blend	9 µl	2.5 U/reaction
PCR Grade Water	to make vol = 810 µl	45 µl/reaction
<b>Final volume</b>	<b>810 µl</b>	

<sup>a)</sup> The tested cDNAs were produced with the Transcriptor First Strand cDNA Synthesis Kit. Five µl of the product mixture is required for each multiplex PCR.

- Dispense 45 µl of Master Mix to each of 17 tubes.
- ▶ To tubes 1 - 16, add 5 µl of one of the buffers (A - P) listed below (all buffers from the PCR Optimization Kit):

pH	Final MgCl <sub>2</sub> Concentration in Reaction (mM)			
	1.0	1.5	2.0	2.5
8.3	A	B	C	D
8.6	E	F	G	H
8.9	I	J	K	L
9.2	M	N	O	P

- ▶ To tube 17, add 5 µl of FastStart High Fidelity Reaction Buffer with MgCl<sub>2</sub> (final MgCl<sub>2</sub> conc. = 1.8 mM).

- Place PCR tubes in the PCR instrument and begin thermal cycling according to the profile in the monoplex procedure above.
- Separate the products (10 µl multiplex reaction/lane) for 1 hour at 100 V on a 1 - 4% Agarose MP gel containing 0.1% ethidium bromide. (For details on what concentration agarose to use, see the monoplex procedure above.)  
 *As a positive control, load a mixture of all individual monoplex PCR products (3 µl each) in one lane of the gel.*
- Determine which buffer performs best (*i.e.*, the buffer that is most suitable for amplifying each of the desired products and, if possible, has the best yield of the products).



### Step 3: Final Optimization of Multiplex PCR with the Best Performing Buffer and Different Additives

After the optimal PCR buffer was identified (in the Step 2 procedure above), the procedure below was used to test the different additives from the PCR Optimization Kit. The results of this step were used to determine whether any of the additives enhanced the yield and specificity of the multiplex PCR. This step required a total of 12 multiplex reactions.

- 1 At room temperature, set up a master mix by adding the components below:
  - ! *The extra volume in the master mix is to compensate for pipetting losses in step 2 below.*

#### Master Mix for All Reactions

Components (Mix 1)	Volume	Final amount (for each reaction)
Best Performing Buffer (from procedure 2), 10× conc.	65 µl	1×
PCR Grade Nucleotide Mix, 10 mM each	13 µl	200 µM each
FastStart High Fidelity Enzyme Blend	6.5 µl	2.5 U/reaction
<b>Final volume</b>	<b>84.5 µl</b>	

- 2 Dispense 6.5 µl of Master Mix to each of 12 tubes.
- 3 To each of the tubes, add:
  - ▶ Template (either 200 ng DNA or 5 µl cDNA)
  - ▶ Multiplex Primer Mix (to make final concentration of each primer in 50 µl reaction = 0.4 µM)
  - ! *To simplify the preparation of these samples, you can prepare a mixture that contains template plus primers (enough for 13 reactions, to allow for pipetting losses), then aliquot the mixture to each of the 12 reaction tubes.*
- 4 Dispense each of the following additives to one tube, as indicated below:

Tube	Additive	Volume to add	Final conc. in reaction
1	PCR Grade Water (control)	2 µl	No additive
2	100% DMSO	1 µl	2% DMSO
3	100% DMSO	2 µl	4% DMSO
4	50% Glycerol	5 µl	5% Glycerol
5	50% Glycerol	10 µl	10% Glycerol
6	50% Glycerol	15 µl	15% Glycerol
7	1% Gelatin	0.5 µl	0.01% Gelatin
8	1% Gelatin	2.5 µl	0.05% Gelatin
9	500 mM (NH <sub>4</sub> ) <sub>2</sub> SO <sub>4</sub>	0.5 µl	5 mM (NH <sub>4</sub> ) <sub>2</sub> SO <sub>4</sub>
10	500 mM (NH <sub>4</sub> ) <sub>2</sub> SO <sub>4</sub>	1 µl	10 mM (NH <sub>4</sub> ) <sub>2</sub> SO <sub>4</sub>
11	500 mM (NH <sub>4</sub> ) <sub>2</sub> SO <sub>4</sub>	2 µl	20 mM (NH <sub>4</sub> ) <sub>2</sub> SO <sub>4</sub>
12	FastStart High Fidelity Enzyme Blend + 25 mM MgCl <sub>2</sub>	0.2 µl Enzyme + × µl 25 mM MgCl <sub>2</sub> <sup>a</sup>	3.5 U Enzyme + 3.5 mM MgCl <sub>2</sub> (total) <sup>a</sup>

- a) The total concentration of enzyme and Mg<sup>2+</sup> includes the amounts added in step 1 above. The amount of MgCl<sub>2</sub> to add depends on the buffer used in step 1. For example if the best performing buffer is buffer A (which adds 1.0 mM MgCl<sub>2</sub>), add an additional 2.5 mM MgCl<sub>2</sub> (5 µl 25 mM MgCl<sub>2</sub>).



- 5 Finally, add enough water to each tube to make the final volume of each = 50  $\mu$ l.
- 6 Place PCR tubes in the PCR instrument and begin thermal cycling according to the profile in the monoplex procedure above.
- 7 Separate the products (10  $\mu$ l multiplex reaction/lane) for 1 hour at 100 V on a 1 – 4% agarose gel containing 0.1% ethidium bromide. (For details on what concentration agarose to use, see the monoplex procedure above.)
  - ! As a positive control, load a mixture of all individual monoplex PCR products (3  $\mu$ l each) in one lane of the gel.
- 8 Determine which additive enhances the yield and specificity of the multiplex PCR.

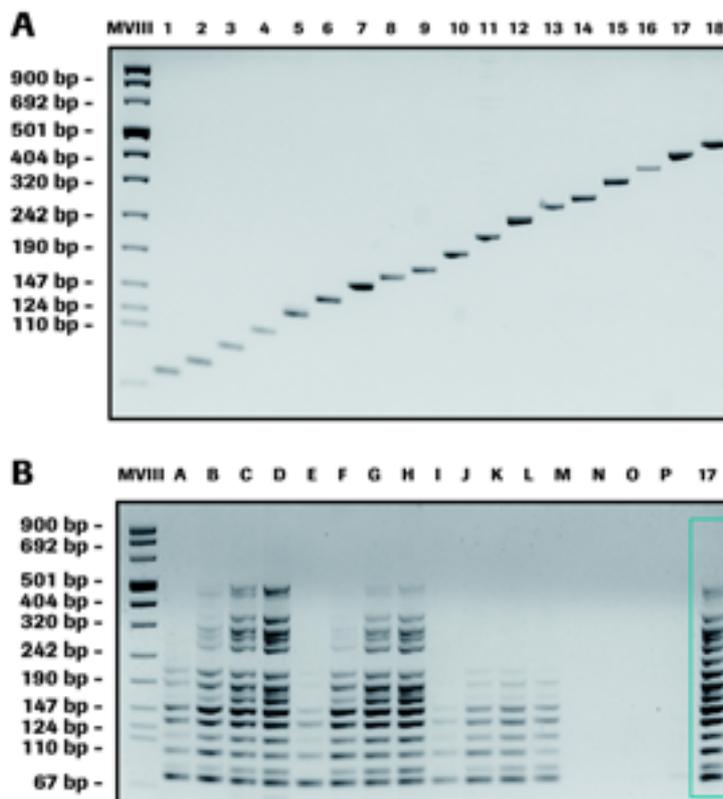
### Results

The three-step protocol above was tested on multiplex PCR systems with nine different primer sets. The results obtained with three of these primer sets is shown below.

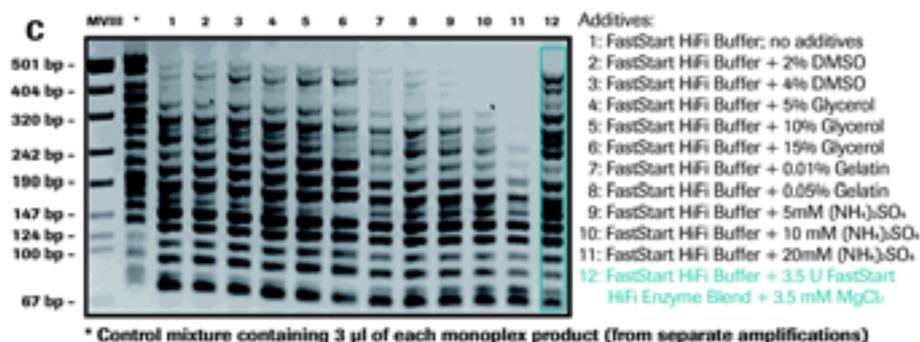


For the results obtained with all nine primer sets, see Schubert et al. (2005).

The most complex primer set (an 18-plex that generated product sizes ranging from 74 to 470 bp) was tested with human genomic DNA. Figure 8.1.1 shows the results of each of the three developmental steps with that primer set. The best results were obtained with FastStart High Fidelity Buffer, extra High Fidelity Enzyme Blend and extra  $MgCl_2$  (panel C, lane 12). In a separate test of sensitivity (results not shown), all 18 products were easily visible on a gel when multiplex PCR was performed with as little as 5 ng genomic DNA template.

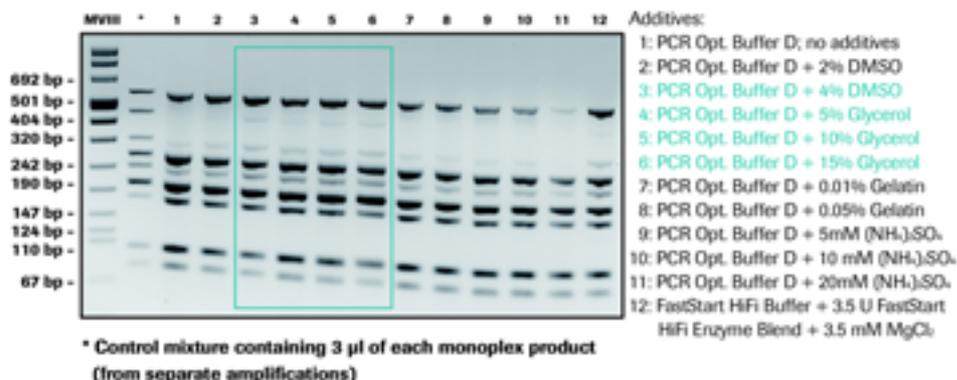


Samples A-P: Buffers from the PCR Optimization Kit  
 Sample 17: FastStart High Fidelity Reaction Buffer



**Figure 8.1.1. Multiplex PCR on human genomic DNA with an 18-plex primer set** (fragment sizes = 74 bp – 470 bp). (A) Monoplex PCR of all products separately. (B) Multiplex PCR with buffers from the PCR Optimization Kit. (C) Multiplex PCR with the best performing buffer (FastStart High Fidelity Buffer) and additives. For panels B and C, the reagent combination(s) that gave optimal results are shown in green type. MVIII: Molecular Weight Marker VIII (RAS).

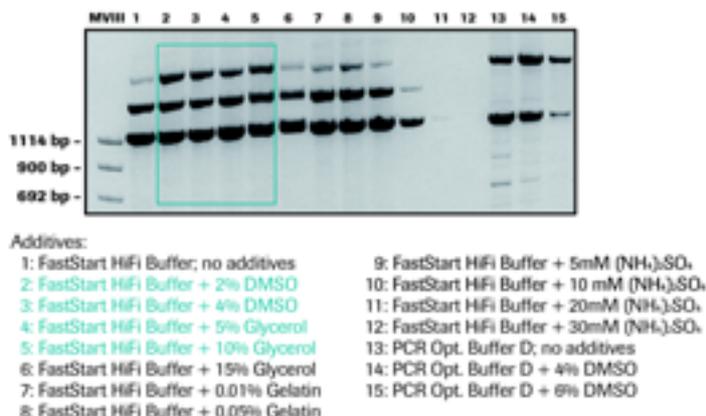
To demonstrate the effectiveness of the protocol on a cDNA template, total RNA from human skeletal muscle was reverse transcribed into cDNA, then the cDNA was used as multiplex PCR template. Figure 8.1.2 shows the results of multiplex PCR with the cDNA. (Only the final optimization step is shown.) For this template and primer combination, the optimal PCR buffer was buffer D (from the PCR Optimization Kit) and the reaction was enhanced by the addition of either 6% DMSO or 5 – 15% glycerol.



**Figure 8.1.2. Multiplex PCR on human cDNA with a 9-plex primer set** (fragment sizes = 89 bp – 540 bp). The cDNA template was obtained by reverse transcription of human skeletal muscle total RNA with the Transcriptor First Strand cDNA Synthesis Kit. Only the results from the final optimization (Step 3 procedure) are shown. The reagent combination(s) that gave optimal results are shown in green type. MVIII: Molecular Weight Marker VIII.



The protocol was also tested for ability to amplify longer sequences. Specifically, a 3-plex primer set that generated products from 1100 bp to 1800 bp long was used to amplify human genomic DNA. Figure 8.1.3 shows the results of this multiplex PCR. (Only the final optimization step is shown.) For this template and primer combination, the optimal PCR buffer was the buffer included in the FastStart High Fidelity PCR System, and the reaction was enhanced by the addition of either 2 – 4% DMSO or 5 – 10% glycerol. In a separate test of sensitivity (results not shown), all 3 products were easily visible on a gel when multiplex PCR was performed with as little as 1 ng genomic DNA template.



**Figure 8.1.3. Multiplex PCR on human genomic DNA with a 3-plex primer set that generates long fragments** (fragment sizes = 1100 bp – 1800 bp). Only the results from the final optimization (Step 3 procedure) are shown. The reagent combination(s) that gave optimal results are shown in green type. MVIII: Molecular Weight Marker VIII.

## Discussion

Any system as complex as multiplex PCR is affected by a number of factors.

Choice of PCR enzyme is obviously crucial. For multiplex PCR, the FastStart High Fidelity Enzyme System offers a number of advantages over single enzymes or other enzyme mixtures. Specifically, the optimized FastStart High Fidelity enzyme mixture transcribes more accurately (up to fourfold higher fidelity) than Taq DNA Polymerase alone and is better able to amplify sequences with high GC content (between 40% and 60%, with the assistance of DMSO).

The optimized enzyme mixture is also very sensitive, since we showed that it could amplify multiple targets from as little as 1 ng human genomic DNA template. In fact, an outside laboratory (Dr. Peterhaensel and colleagues, RWTH, Aachen, Germany, as reported in Schubert *et al.*, 2005) was able to obtain even higher sensitivity using a slightly modified protocol, a plant DNA template and a 5-plex primer set (product lengths, 259 bp – 847 bp). In their hands, the FastStart High Fidelity Enzyme System was able to amplify all five targets from as little as 0.375 ng template.

Successful multiplex PCR also depends greatly on the sizes of the products. It is difficult to amplify very long and short products at the same time with equivalent yields, because the polymerase will amplify the shorter products more efficiently. Therefore, we usually designed primers to produce amplicons within one of these three optimal size ranges: 50 – 600 bp, 450 – 1100 bp, or 1100 – 1800 bp.

The purity of the primers also affects results. Only HPLC-purified primers should be used in a multiplex system! Less purified primer preparations may contain shorter oligonucleotide fragments that can contribute nonspecific bands to the final product mixture, greatly complicating interpretation of the results.

Reaction additives, and particularly cosolvents were frequently found to enhance multiplex PCR results. Of the additives tried, we found DMSO and glycerol to be the most effective. Reportedly (Landre *et al.*, 1995), DMSO reduces nonspecific priming and secondary structure (*e.g.*, of GC-rich sequences), and facilitates strand separation by lowering the  $T_m$  of the target. Glycerol makes the Taq DNA Polymerase more resistant to heat damage, thereby increasing product yield (Aoyagi, K., 2001).

Finally, successful generation of cDNA templates requires use of an anchored oligo(dT) primer, rather than a hexamer primer. Hexamer primers can interfere with the subsequent multiplex PCR.

**Products from Roche Applied Science used to generate these results:**

FastStart High Fidelity PCR System, PCR Optimization Kit, PCR Grade Nucleotide Mix, Agarose MP, DNA Molecular Weight Marker VIII, PCR Grade Water, LightCycler® Probe Design Software 2.0, Transcriptor First Strand cDNA Synthesis Kit.



*For information on ordering these products, see the Appendix.*



## 8.2 Specific Amplification of Difficult PCR Products from Small Amounts of DNA Using FastStart Taq DNA Polymerase

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### Introduction

The availability of a specific, clean polymerase chain reaction (PCR) product in sufficient amounts prior to cycle sequencing is one important prerequisite for the generation of a good-quality DNA sequence. Since heterozygote samples must be unambiguously identified, excellent DNA quality is particularly crucial for applications such as mutation detection or discovery of single nucleotide polymorphisms (SNPs).

As the PCR efficiency is controlled by many parameters – such as polymerase type, buffer type, primer concentration and stability, dNTP purity and concentration, cycling parameters, as well as complexity and concentration of starting template – it is not possible to establish one standard PCR amplification protocol. Therefore, it is necessary to optimize PCR conditions for each PCR amplicon.

For example, successful amplification of difficult templates (*e.g.*, templates containing GC-rich regions) often requires the addition of cosolvents (*e.g.*, dimethylsulfoxide [DMSO]) to lower the DNA-strand separation temperature. However, this method has the drawback that it may inhibit enzyme activity and slow the extension rate – which is a problem if only a small amount of DNA is available.

Here, we have compared the ability of Taq DNA Polymerase and FastStart Taq DNA Polymerase to amplify difficult (GC-rich) DNA templates in the presence and absence of cosolvents, starting with different amounts of DNA.

### Materials and Methods

A 540-bp DNA fragment from 5-hydroxytryptamine receptor 2C (HTR2C; GC-content, 65%) was amplified in a total volume of 50  $\mu$ l using either Taq DNA Polymerase or FastStart Taq DNA Polymerase. Reactions were performed in a thermal cycler (MJ Research Tetrad, Watertown, MA).

Human genomic DNA (20 ng) was amplified using 0.2 mM of each primer (5'-CAGC-CATCCGGGACCTGTC-3' and 5'-ACCTGCCGATTGCATATGAAC-3') in the presence of the buffer supplied with the enzyme (for Taq DNA Polymerase: 10 mM Tris pH 8.3, 50 mM KCl, 1.5 mM MgCl<sub>2</sub>; for FastStart Taq DNA Polymerase: 50 mM Tris pH 8.3, 10 mM KCl, 2.0 mM MgCl<sub>2</sub>, 5 mM [NH<sub>2</sub>SO<sub>4</sub>], 200 mM of each dNTP, and 1.5 units enzyme. The concentration of DMSO or GC-rich solution (provided with FastStart Taq DNA Polymerase) ranged from 0–10%.

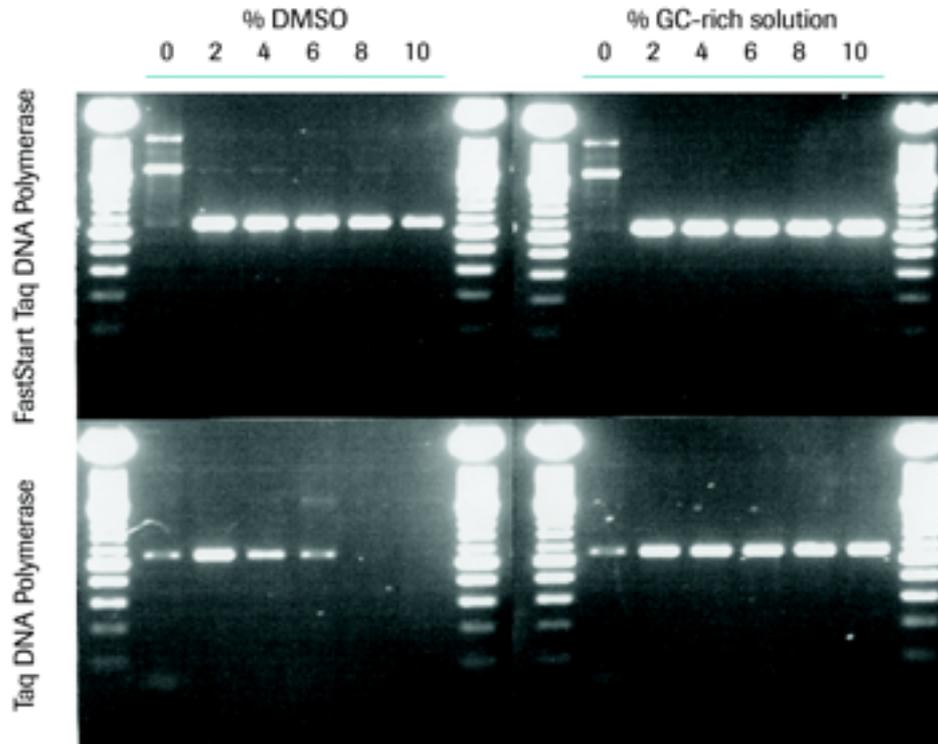
The thermal cycling protocol was: 15 minutes initial incubation at 95 °C, followed by 35 cycles of PCR (1 minute denaturation at 94°C, 30 seconds annealing at 60°C, 1 minute extension at 72 °C), and 10 minutes final extension at 72 °C.

The resulting 540-bp PCR products were electrophoresed through a 1.5% agarose gel containing ethidium bromide, then visualized under UV light.



## Results and Discussion

In reactions with either Taq DNA Polymerase or FastStart Taq DNA Polymerase, it was not possible to obtain the desired 540-bp amplicon in sufficient amounts without adding DMSO or GC-rich solution.

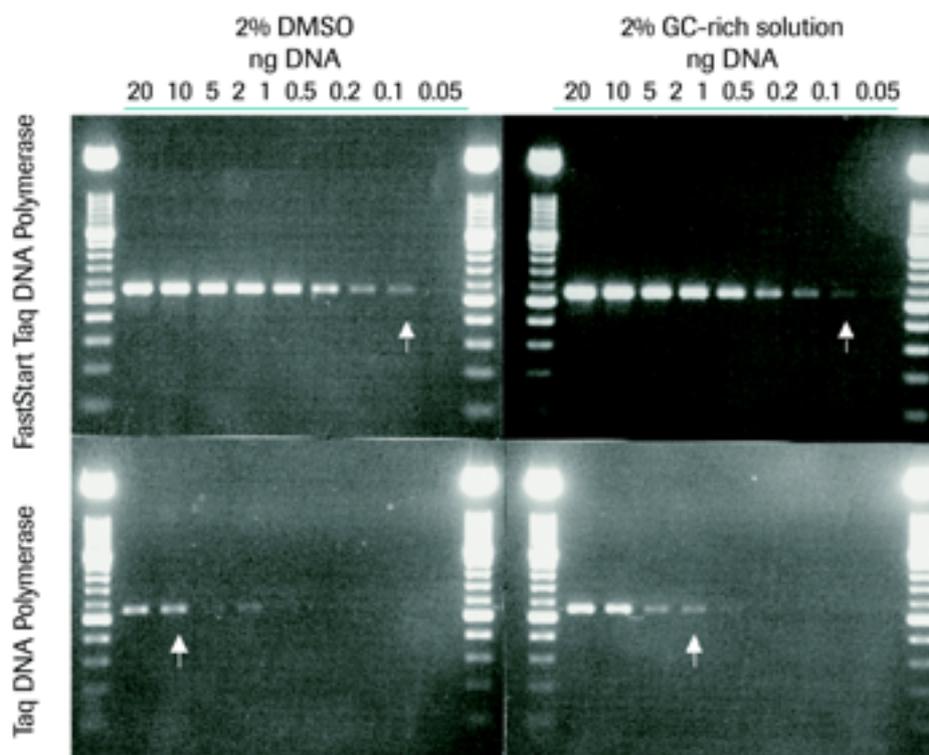


**Figure 8.2.1.** Amplification of a 540-bp fragment of the hydroxytryptamine receptor 2C (with a GC content of 65%) using FastStart Taq DNA Polymerase and Taq DNA Polymerase. Different amounts of DMSO or GC-rich solution were added.

The addition of DMSO and GC-rich solution improved both the specificity and yield of the reactions (Figure 8.2.1). Addition of either 2% DMSO or 2–10% GC-rich solution allowed both enzymes to amplify a specific PCR product, but FastStart Taq DNA Polymerase produced a higher yield. Therefore, FastStart Taq – unlike conventional Taq DNA Polymerase – does not have a slower extension rate in the presence of DMSO or GC-rich solution. High concentrations (more than 2%) of DMSO inhibit the activity of Taq DNA Polymerase, but do not inhibit the activity of FastStart Taq DNA Polymerase.

FastStart Taq DNA Polymerase, when used with the specially developed GC-rich solution, seems to be the most suitable enzyme for templates with a high (65%) GC content.





**Figure 8.2.2. Dilutions of human genomic DNA were amplified in the presence of 2% DMSO or 2% GC-rich solution.** FastStart Taq DNA Polymerase showed greater sensitivity (*i.e.*, a visible PCR product was obtained from only 100 pg of starting DNA). Arrows mark the threshold of detection for each enzyme-cosolvent combination.

To determine the sensitivity of both enzymes, dilutions of human genomic DNA (50 pg, 100 pg, 200 pg, 500 pg, 1 ng, 2 ng, 5 ng, 10 ng, 20 ng) were amplified in the presence of 2% DMSO or 2% GC-rich solution (Figure 8.2.2). The threshold of detection is defined as the amount of starting DNA that generates a visible PCR signal (*i.e.*, presence of the 540-bp fragment on the gel; see arrows). Taq DNA Polymerase produced a visible PCR product in 2% DMSO from as little as 10 ng template DNA, and in 2% GC-rich solution from as little as 2 ng DNA. FastStart Taq DNA Polymerase showed at least tenfold greater sensitivity, generating visible PCR products from only 100 pg of DNA template. These results are in agreement with the results obtained with different concentrations of cosolvents.

The higher sensitivity of FastStart Taq DNA Polymerase can be explained by its robustness in the presence of additives (*e.g.*, DMSO or GC-rich solution). FastStart Taq DNA Polymerase did not seem to be inhibited by these cosolvents, nor did the solvents slow the extension rate of the enzyme.

### **Summary**

We have successfully used FastStart Taq DNA Polymerase to obtain clean, specific PCR products, suitable for sequencing, from small amounts (as little as 100 pg) of GC-rich template DNA. In addition to the advantage of higher specificity offered by a hot start enzyme, FastStart Taq Polymerase has an additional advantage; it is not inhibited even by high concentrations of cosolvents (DMSO and GC-rich solution). Therefore it is superior to Taq DNA Polymerase for this application.

Reprinted from Biochemica 4/2002, pages 25-26, October 2002 © Springer-Verlag 2002.

### **Products from Roche Applied Science used to generate these results:**

Taq DNA Polymerase; FastStart Taq DNA Polymerase.



*For information on ordering this product, see the Appendix.*



## 8.3 FastStart Taq DNA Polymerase Is Ideally Suited for RT-PCR of Laser Captured Microdissected Material

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### Introduction

Laser capture microdissection (LCM) is a new technique allowing the isolation of small numbers of cells or cell subpopulations from fixed tissue sections (Emmert-Buck *et al.*, 1996). Downstream applications can include gene expression studies based on the sensitive reverse transcription-polymerase chain reaction (RT-PCR) technique. However, if only small quantities of starting material are available, it is important to amplify the genes with great specificity and sensitivity. We have therefore undertaken a comparative study of commercially available reverse transcriptases and hot start Taq DNA Polymerases to identify the optimal enzyme combination for quantification of gene expression in a small quantity of cells isolated by LCM.

### Material and Methods

The PixCell II Laser Capture Microdissection System (Arcturus Engineering) was used to procure separate populations of mouse luminal epithelial cells from 8- $\mu$ m frozen sections of uterus fixed in 70% ethanol. An average of 100 laser shots (15  $\mu$ m) per sample was used, yielding approximately 100-200 cells. Total RNA was extracted from each sample with a commercially available kit, then divided into three equal parts.

cDNA was synthesised using random hexamers and three different reverse transcriptases (one each from suppliers A, B, and C) in a total volume of 20  $\mu$ l. Each reaction was performed according to the manufacturer's instructions. The expression of glyceraldehyde- 6-phosphate dehydrogenase (GAPDH) was determined by PCR amplification of 1  $\mu$ l cDNA in the presence of these primers: 5'-ACCCAGAAGACTGTGGATGG-3' and 5'-GAGACAACCTGGTCCTCAG-3'.

GAPDH was amplified in a thermal cycler using five different hot start Taq DNA Polymerases (one each from suppliers 1, 2, 3, and 4; FastStart Taq DNA Polymerase from Roche Applied Science). Each reaction (total volume, 20  $\mu$ l) contained the PCR buffer supplied by the respective manufacturer, 0.2  $\mu$ M of each primer and 200  $\mu$ M of dNTP.

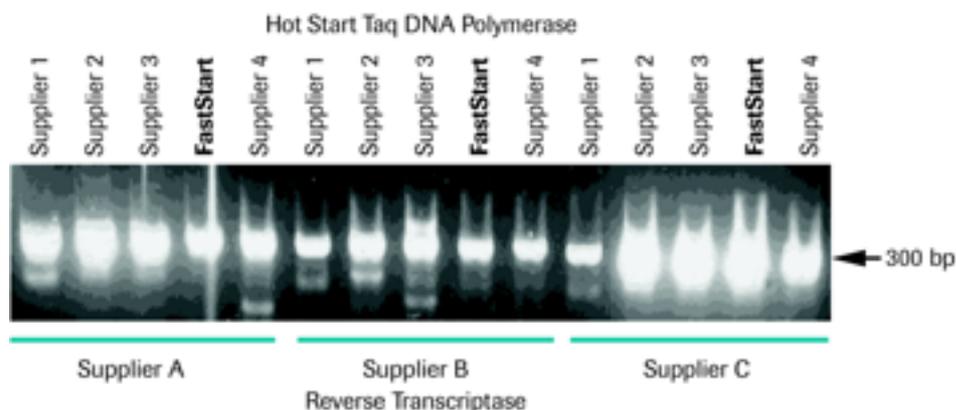
The reaction with FastStart Taq DNA Polymerase contained 0.8 unit of enzyme and a final MgCl<sub>2</sub> concentration of 2 mM. Thermal cycling conditions were: an initial 4-minute denaturation/activation step at 95 °C, followed by 40 PCR cycles (30 second denaturation at 95°C, annealing at 60°C, and 45 second elongation at 72°C), and a final 7 minute extension at 72°C. Similar cycling conditions were used for the other hot start Taq DNA Polymerases, while the reaction mixtures were prepared according to the manufacturers' instructions. The resulting 300-bp PCR products were electrophoresed in parallel through a 2% agarose gel in the presence of a SYBR green stain, then visualised under UV light.



## Results and Discussion

To compare the efficiency of several reverse transcriptases and hot start Taq DNA Polymerases in two-step RT-PCRs, total RNA was extracted from luminal epithelial LCM samples of a mouse uterus. Three different reverse transcriptases were used for cDNA synthesis. Five different hot start Taq DNA Polymerases, including FastStart Taq DNA Polymerase, were used for subsequent PCR amplification of GAPDH. PCR amplifications were performed in parallel.

GAPDH cDNA was amplified in each case but the different combinations of reverse transcriptases and hot start Taq DNA Polymerases produced varying degrees of efficiency and specificity (Figure 8.3.1). The amount of GAPDH amplification with FastStart Taq DNA Polymerase was generally as good as or better than amplification with hot start Taq DNA Polymerases from other suppliers. However, non-specific amplification was detected in the majority of PCRs that used hot start Taq DNA Polymerase from other suppliers, whereas it was minimal in PCRs that used FastStart Taq DNA Polymerase. FastStart Taq DNA Polymerase was therefore chosen for experiments involving PCR amplification of RNA extracted from LCM material.



**Figure 8.3.1. Comparison of reverse transcriptase and hot start Taq DNA Polymerase efficiency in two-step RT-PCRs.** cDNA synthesized by each of three different reverse transcriptases was amplified in parallel reactions with each of five different hot start DNA polymerases. The template RNA was obtained from mouse uterine luminal epithelium cells isolated by laser capture microdissection. The 300-bp PCR product, a GAPDH amplicon, was obtained after 40 PCR cycles.

FastStart Taq DNA Polymerase has also successfully amplified targets present at a lower abundance than GAPDH in LCM material, such as estrogen receptors alpha and beta (results not shown).

## Summary

We have successfully used FastStart Taq DNA Polymerase to amplify gene expression targets from limited amounts of cells that were isolated by laser capture microdissection. This product provides superior sensitivity and less nonspecific amplification than other hot start Taq DNA Polymerases.

Reprinted from *Biochemica* 2/2002, pages 24-25, April 2002 © Springer-Verlag 2002.

## Product from Roche Applied Science used to generate these results:

FastStart Taq DNA Polymerase.



For information on ordering this product, see the Appendix.



## 8.4 Cloning of mRNAs and Rapid Screening by Direct Colony PCR with the FastStart PCR Master

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### Introduction

Often cloning experiments require screening to find a recombinant plasmid that has the desired orientation, insert length, etc. Direct colony PCR offers a fast screening method that circumvents the need for template purification before PCR and allows the rapid characterization of multiple clones.

For this application a robust but specific and sensitive PCR system is needed. The Roche Applied Science FastStart PCR Master (a ready-to-use, 2× concentrated reaction mix for hot-start PCR) provides such a system for direct colony PCR.

In this paper, we describe the use of the FastStart PCR Master for the amplification and cloning of mRNA. An overview of the workflow for the technique, which includes direct colony screening, is presented in Figure 8.4.1.



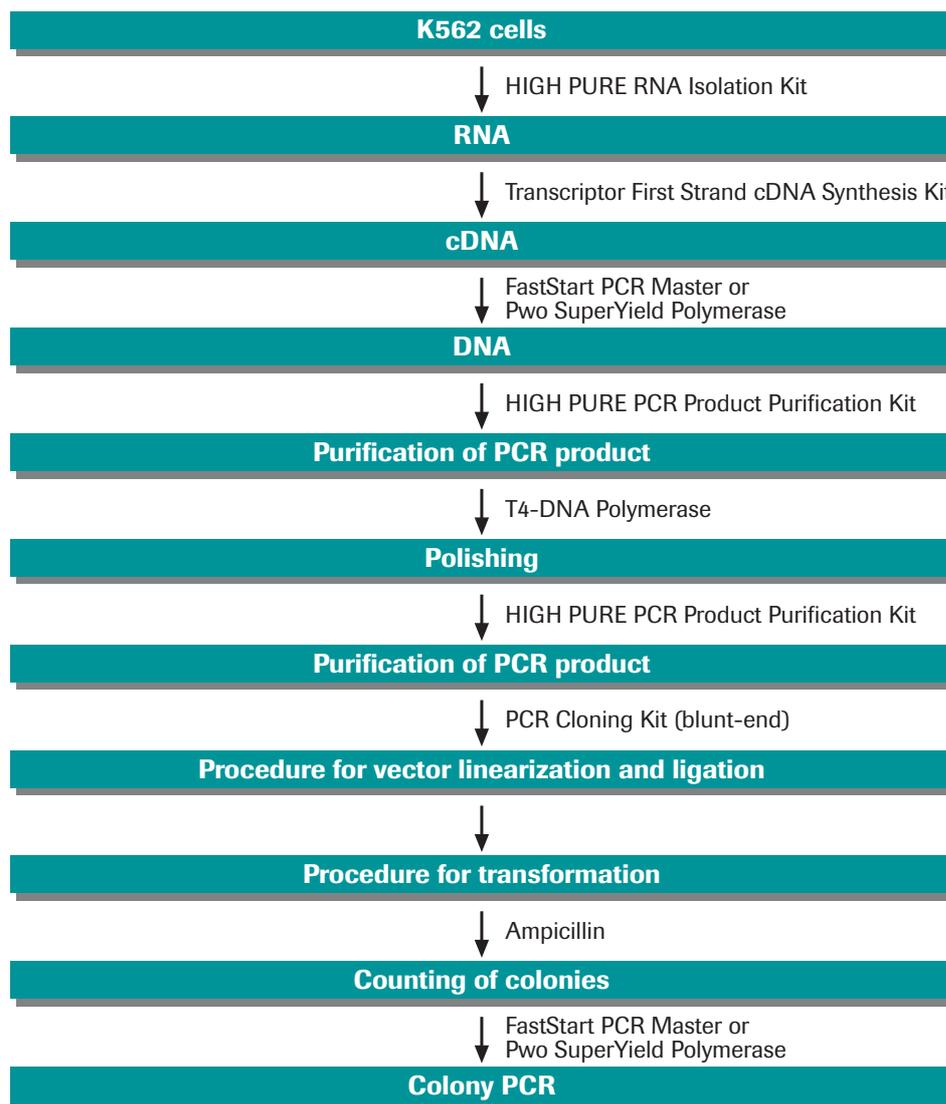


Figure 8.4.1. Workflow strategy.

### Materials and Methods

All reagents (except for growth media and competent cells), enzymes and kits were supplied by Roche Applied Science.

#### Purification of Total RNA

Total RNA was obtained from K562 cells with the HIGH PURE RNA Isolation Kit as described in the package insert. Cells were resuspended in 200  $\mu$ l PBS and lysed by addition of 400  $\mu$ l Lysis-Binding Buffer and vortexing. The lysate was transferred to a HIGH PURE Filter Tube and the tube was centrifuged in a microfuge for 15 seconds at 8000  $\times$  g. The RNA, which was bound to the glass fleece filter in the Filter Tube, was treated with DNase I (15 minutes, 15 to 25°C). The filter was washed with successive 500  $\mu$ l aliquots of Wash Buffer I and Wash Buffer II, and the Filter Tube was centrifuged (15 seconds, 8000  $\times$  g) after each wash. The filter was washed a final time with an additional 200  $\mu$ l of Wash Buffer II, and the liquid was removed by high speed centrifugation (2 minutes; 13,000  $\times$  g). PCR Grade Water (50  $\mu$ l) was added to the Filter Tube and the RNA was eluted from the Filter Tube by centrifugation (1 minute, 8000  $\times$  g). The purified total RNA was collected in a clean, sterile 1.5 ml microfuge tube.



### Synthesis and Amplification of cDNA

An aliquot of the isolated RNA was reverse transcribed to cDNA with the Transcriptor First Strand cDNA Synthesis Kit, as described in this manual (Chapter 5, section 5.4.3.1). Random hexamer primers and a conventional thermal cycler were used.

In the second step of the two-step RT-PCR procedure, 6 µl of cDNA was amplified with the FastStart PCR Master. For comparison, 6 µl of the cDNA was also amplified with Pwo SuperYield DNA Polymerase. The PCRs were performed according to the package insert of each polymerase, except that the total volume of each reaction was increased threefold (to 150 µl). The desired target of the amplification was a 380-bp fragment of the human interleukin gene (IL-2). Gene-specific primers (5'-CTCACCAGGATGCTCAC, 3'-GTAGCAAACCATACATT, 4.5 µl of each primer) were used for each PCR.

Each reaction was cycled according to the following parameters: 2 minutes initial denaturation at 94°C; 10 cycles of PCR according to program 1 (30 seconds at 94°C, 30 seconds at 55°C, 45 seconds at 68°C); and an additional 30 cycles of PCR according to program 2 [times and temperatures as in program 1, but extension time was increased by 5 seconds per cycle (*i.e.*, 50 seconds at 68°C at the beginning of program 2, but 195 seconds by the end of program 2)]. The final extension was for 7 minutes at 68°C.

To verify the success of the PCR, an aliquot (15 µl) of each reaction was analyzed on a 3% Agarose MP gel. PCR products were visualized with 1 µg/ml ethidium bromide and compared with DNA Molecular Weight Marker VIII (Figure 2).

### Purification and Cloning of PCR Product

The PCR product from each reaction was purified with the HIGH PURE PCR Product Purification Kit as described in the package insert. The purification procedure was functionally very similar to the RNA purification described above, since it also involved a HIGH PURE Filter Tube, but required a DNA-specific Wash Buffer and different centrifugation times.

Subsequently, the PCR product generated with the FastStart PCR Master was polished with T4 DNA Polymerase to generate blunt-ended PCR fragments. The polishing procedure was as follows: 1 U of T4 DNA Polymerase was incubated with 300 ng of the PCR product, reaction buffer and dNTPs for 30 minutes at 37°C. Subsequently, the polished PCR product was again purified with the HIGH PURE PCR Product Purification Kit.

Note: Since Pwo SuperYield DNA Polymerase generates blunt-ended products, the PCR product generated with Pwo SuperYield DNA Polymerase did not require polishing.

The purified amplification product from each PCR was cloned into a pCAP<sup>s</sup> cloning vector with the PCR Cloning Kit (blunt-end). Vector restriction and ligation were performed according to the package insert of the kit. Restriction (with *Mlu* NI from the kit) required 5 minutes incubation at 37°C, followed by 15 minutes enzyme inactivation at 65°C. Ligation (with T4 DNA ligase) required 5 minutes incubation at 15 - 25°C.



### Transformation and Plating

Each cloned PCR product was transformed, using the procedure from the package insert of the PCR Cloning Kit (blunt-end), into competent *E. coli* DH5-alpha cells. Briefly, the procedure involves these steps: (1) incubation of competent cells with culture medium on ice for 10 minutes, (2) addition of ligation reaction mix (used directly without purification) to competent cells followed by incubation for 30 minutes on ice, (3) incubation of the DNA-cell mix for 45 seconds at 42°C followed by an immediate 2 minute incubation on ice, and (4) addition of fresh medium followed by incubation for 30 minutes at 37°C.

Different amounts (50 – 100 µl) of each transformation mixture were plated on selective LB plates that had been supplemented with 100 µg/ml Ampicillin. The plates were incubated for 16 – 18 hours at 37°C. On the next morning, the colonies on each plate were counted.

### Direct Colony PCR

Ten clones derived from each cloned PCR product were randomly picked with sterile toothpicks. To lyse the cells and release the plasmid DNA, each picked colony was heated in 100 µl H<sub>2</sub>O for 5 minutes at 100°C. Cell debris was pelleted by centrifugation at 13,000 rpm for 1 minute.

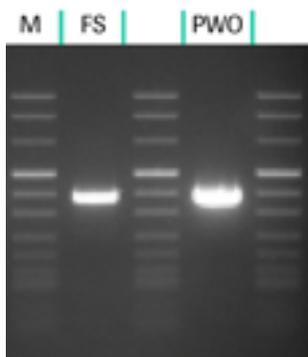
An aliquot (5 µl) of each supernatant was used for direct colony PCR, which was performed with the FastStart PCR Master Mix and gene-specific primers. PCR cycling parameters were as follows: 2 minutes initial denaturation at 94°C; 10 cycles of PCR according to program 1 (30 seconds at 94°C, 30 seconds at 55°C, 45 seconds at 68°C); and an additional 20 cycles of PCR according to program 2 [times and temperatures as in program 1, but extension time increased by 5 seconds per cycle (*i.e.*, 50 seconds at 68°C at the beginning of program 2, but 145 seconds by the end of program 2)]. The final extension was for 7 minutes at 68°C.

A 15 µl aliquot of the amplification product from each clone was loaded directly on a 2% agarose gel for electrophoresis and product analysis (Figure 3).



## Results and Discussion

### PCR for Template Amplification

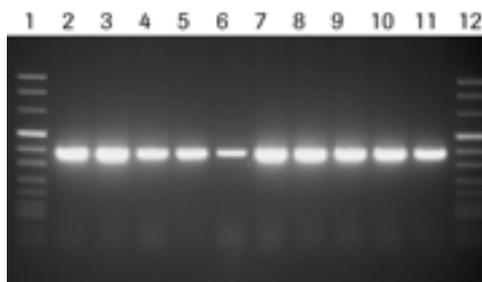


**Figure 8.4.2. Performance comparison.** The 380-bp fragment of the IL-2 gene was amplified using either FastStart Master Mix (FS) or Pwo SuperYield DNA Polymerase (PWO) according to the package insert of each polymerase. A 15  $\mu$ l aliquot of each PCR product was analyzed on a 3% agarose gel. The amplification efficiency of the FastStart Master Mix was comparable to that of the Pwo SuperYield DNA Polymerase. M, Molecular Weight Marker VIII.

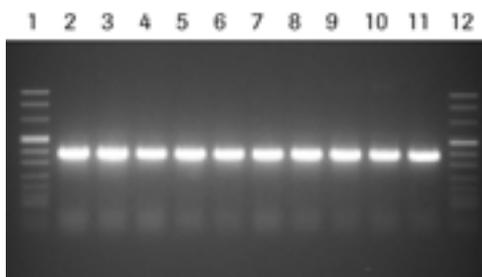
Agarose gel analysis showed that the polymerases consistently amplified a 380-bp fragment (Figure 2). The FastStart PCR Master and the Pwo SuperYield DNA Polymerase yielded comparable amounts of amplification product.

### PCR for Colony Screening

More than 50 colonies were obtained from each cloning experiment. Comparable numbers of colonies were obtained from cloned PCR products that were generated with the two DNA polymerases.



**Figure 8.4.3. Colony PCR of clones that contained plasmid and inserts generated with the FastStart PCR Master.** Direct colony PCR with the FastStart PCR Master was used to amplify the cloned 380-bp fragment from the IL-2 gene. A 15  $\mu$ l aliquot of the amplification product from each clone was analyzed on a 2% agarose gel. The gel shows that all randomly selected colonies carried the ligation product.



**Figure 8.4.4. Colony PCR of clones that contained plasmid and inserts generated with Pwo SuperYield DNA Polymerase.** Direct colony PCR with the FastStart PCR Master was used to amplify the cloned 380-bp fragment from the IL-2 gene. A 15  $\mu$ l aliquot of the amplification product from each clone was analyzed on a 2% agarose gel. The gel shows that all randomly selected colonies carried the ligation product.

The FastStart PCR Master was used for direct colony PCR. All 20 randomly selected clones contained the 380-bp insert ligated into the plasmid vector pCAP<sup>s</sup> (Figures 3, 4). Consequently, the cloning experiment succeeded regardless of the polymerase used to amplify the original cDNA and produce the 380-bp insert.

Pwo SuperYield DNA Polymerase is a proofreading polymerase while FastStart Taq DNA is not. Consequently, Pwo SuperYield DNA Polymerase has 18-fold higher transcriptional fidelity than FastStart Taq DNA Polymerase (and the FastStart PCR Master, which contains the FastStart Taq DNA Polymerase). However, the results show that both the FastStart PCR Master and Pwo SuperYield DNA Polymerase generated amplification products that were highly suitable for cloning.

Furthermore, the FastStart PCR Master was shown to be extremely suitable for PCR performed directly on *E. coli* cells. This master mix was the only reagent needed for high-throughput screening of the colonies.

Colony PCR is a handy technique for simplifying the detection of successful cloned inserts. It has a highly efficient workflow and replaces time-consuming conventional clone analysis. The results clearly demonstrated that the FastStart PCR Master is an ideal hot-start tool for both the PCR steps in colony PCR (*i.e.*, cDNA amplification and direct colony screening).

The figures in this paper were reprinted from a shorter version of this article that appeared in *Biochemica* 1/2006, pages 14-16, January 2006 © Springer-Verlag 2006.

**Products from Roche Applied Science used to generate these results:**

HIGH PURE RNA Isolation Kit, Transcriptor First Strand cDNA Synthesis Kit, FastStart PCR Master, Pwo SuperYield DNA Polymerase, HIGH PURE PCR Product Purification Kit, PCR Cloning Kit (blunt-end), T4 DNA Polymerase, PBS Buffer, PCR Grade Water, Protector RNase Inhibitor, PCR Grade Nucleotides, Molecular Weight Marker VIII, Agarose MP, Ampicillin.



*For information on how to order this product, see the Appendix.*



## 8.5 FastStart High Fidelity PCR System Simplifies Study of Epigenetics and DNA Methylation

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### Introduction

Epigenetic mechanisms such as DNA methylation, histone acetylation, and small RNA interference, and their effects on gene activation and inactivation are important in phenotype imprinting, development, and cancer biology (Soejima *et al.*, 2004). Abnormal methylation of critical genes may lead to severe developmental diseases such as Angelman or Prader-Willi syndrome, and to the development of cancer (Walter and Paulsen, 2003). The recent interest in clinical aspects of epigenetics has paralleled the emergence of new techniques for studying DNA methylation (Tollefsbol, 2004).

Methylated nucleotides can easily be detected by Southern blotting of DNA digested with methylation-specific restriction enzymes. However, not all methylated positions fall within restriction enzyme sequences; moreover, this technique requires substantial amounts of DNA. These problems can be overcome by using methylation-specific PCR (M-PCR) or by sequencing deaminated DNA. In M-PCR, the unmethylated cytosines in a given template DNA are deaminated to uracil with bisulfite, thus changing a CG to an AT pair (Herman *et al.*, 1996). Methylated cytosines are resistant to deamination.

To distinguish between methylated and unmethylated genes or promoters, methylation-specific primers for different sizes of amplicons are used. The amplification of a single gene copy by PCR is highly sensitive, and – with stringent template handling and primer design – highly specific. The main obstacles to a wider use of M-PCR are difficulties in DNA deamination, purification of deaminated DNA, and laborious, expensive optimization (Cottrell and Laird, 2003).

We have devoted a substantial amount of time, resources, and energy to using M-PCR in the study of male infertility genetics in our laboratory. We would like to share our experiences with the FastStart High Fidelity PCR System, which seems to be optimal for M-PCR.

We tested the hypothesis that the methylation pattern (inactivation) of the X chromosome-inactivating transcript (XIST) located on the X chromosome in females (46, XX) and males with Klinefelter syndrome (KS) (47, XXY) is the same (Hall and Lawrence, 2003).

### Materials and Methods

DNA from peripheral blood lymphocytes of healthy males and females, and from men with KS was extracted using commercially available kits. The DNA was stored at  $-20^{\circ}\text{C}$ .



### DNA Deamination

DNA (0.5–1 mg in 50  $\mu$ l H<sub>2</sub>O) was denatured at 37°C (17 minutes) and 55°C (2 minutes) with 5.5  $\mu$ l of 2 M NaOH. Subsequently, 30  $\mu$ l of 10 mM hydroquinone and 520  $\mu$ l of 3 M sodium hydrogen sulfite, both freshly prepared, were added to the DNA solution and the solution was overlaid with mineral oil. The mixture was incubated in a water bath at 55°C overnight (in darkness). The reaction was terminated by adding NaOH during the purification process. Deaminated DNA was suspended in Tris-EDTA and stored at -20°C.

### Methylation-Specific PCR

We used methylation-specific primers for the gene of interest (XIST) and for a control gene with an opposite pattern of methylation (familial mental retardation gene 1, FMR1) (Zalfa and Bagni, 2004).

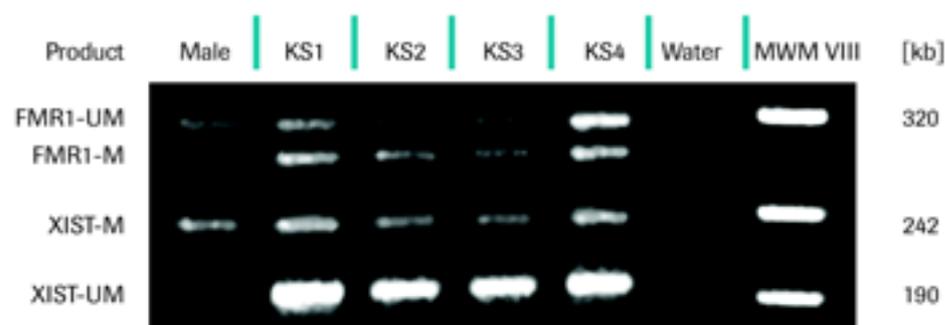
For reactions with FastStart Taq DNA Polymerase, the PCR master mix contained (for each reaction): 2.5  $\mu$ l 10x buffer mix (final Mg<sup>2+</sup> concentration, 1.8 mM), 0.5  $\mu$ l dNTPs, 0.25  $\mu$ l FastStart High Fidelity Polymerase, and enough water to make the final reaction volume 25  $\mu$ l. DNA template (200 ng in 4  $\mu$ l water) was mixed with 2  $\mu$ l primer multiplex mix. The PCR master mix was added to the DNA and primer mix. PCR was performed in a thermal cycler using the following settings: 1  $\times$  3 minutes at 95°C, followed by 40 cycles  $\times$  (95°C/30 seconds, 61°C/40 seconds, 72°C/50 seconds), followed by 1  $\times$  7 minutes at 71°C, then 4°C.

The PCR master mixes for other polymerases tested were prepared according to manufacturers' instructions.

Some authors suggested using specialized M-PCR reaction buffer with 0.1% Triton-X and ammonium sulfate (5–30 mM); however, in our experiment, the addition of Triton-X or ammonium sulfate had a negative impact on the yield obtained with all polymerases tested.

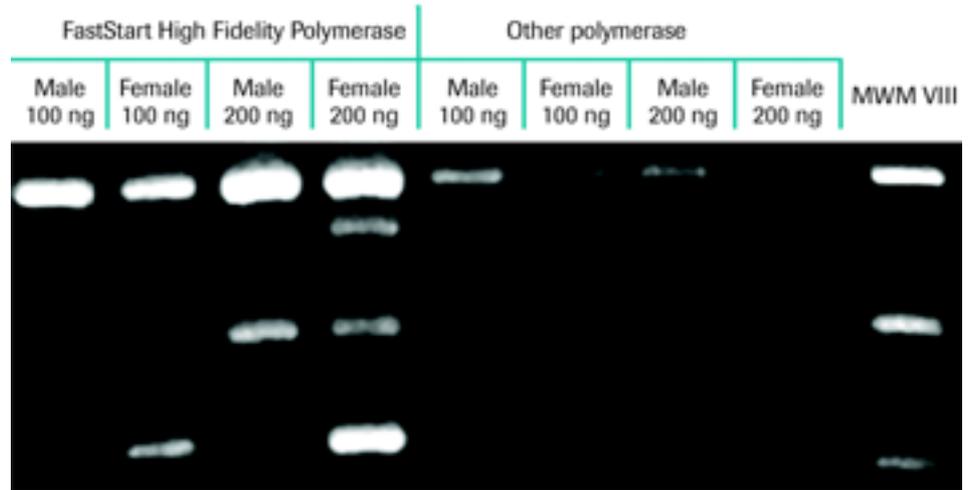
### Results

Methylation-specific PCR was 100% specific and 100% sensitive in detecting X chromosome polysomy in males with nonmosaic KS. The DNA from males showed two bands on an agarose gel: one for the unmethylated FMR1 promoter (FMR1-UM) and one for the methylated XIST gene (XIST-M) (Figure 8.5.1).

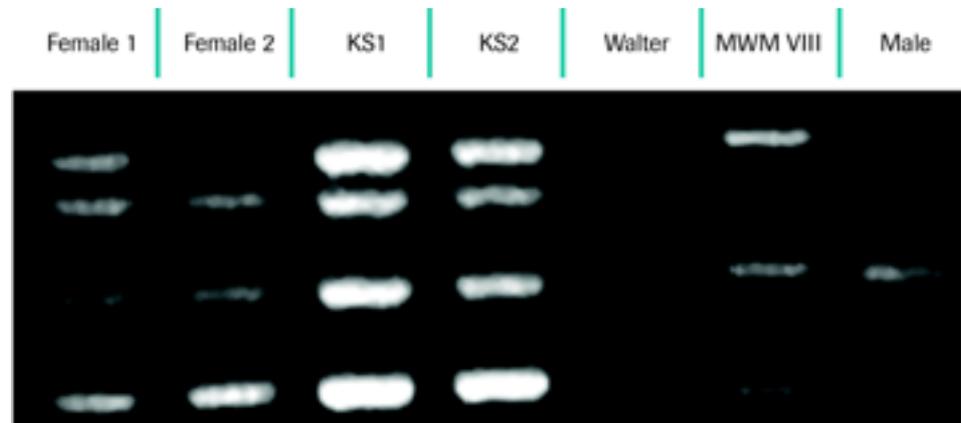


**Figure 8.5.1. The FastStart High Fidelity PCR System yields consistent results in M-PCR.** For each lane, 10  $\mu$ l of DNA were mixed with 2  $\mu$ l of loading buffer and loaded on a 4% NuSieve 3:1 agarose gel. A healthy male has only one X chromosome, thus M-PCR shows only one band for methylated XIST (XIST-M). The FMR1 gene has an opposite methylation pattern and serves as a control. (KS1, KS2, KS3, KS4 – template DNA from subjects with Klinefelter syndrome)

The DNA from females and males with KS showed four bands: methylated and unmethylated FMR1 promoter (FMR1-UM, FMR1-M), and methylated and unmethylated XIST gene (XIST-M, XIST-UM, Figures 8.5.1 – 8.5.3). The pattern of XIST methylation in men with KS was the same as that in females (Figure 8.5.3).



**Figure 8.5.2. Comparison with other polymerases using the same template DNA:** For each lane, 10  $\mu$ l of DNA were mixed with 2  $\mu$ l of loading buffer and loaded on a 4% NuSieve 3:1 agarose gel. FastStart High Fidelity Polymerase yields superior results in M-PCR. No optimization steps were necessary.



**Figure 8.5.3. Methylation pattern.** For each lane, 10  $\mu$ l of DNA were mixed with 2  $\mu$ l of loading buffer and loaded on a 4% NuSieve 3:1 agarose gel. Analysis using FastStart High Fidelity Polymerase shows that men with KS (KS1, KS2) have the same pattern of XIST methylation as females (female 1, female 2).

When compared with other available polymerases (Figure 8.5.2), the FastStart High Fidelity PCR System yields the best results in methylation-specific PCR.

### **Conclusions**

The FastStart High Fidelity PCR System is a highly reliable and cost efficient option for research applications that involve methylation-specific PCR. The FastStart High Fidelity PCR System has worked “out of the box” with minimal optimization.

Adequate deamination and careful primer design are necessary for success. In multiplex M-PCR, the primer concentrations must be established empirically, especially for semi-quantitative analysis of methylation patterns.

In our laboratory, we have expanded this technique to analysis of other genes involved in male infertility and the results show excellent reproducibility. Based on this report, we strongly believe that M-PCR using the FastStart High Fidelity PCR System has potential for new applications in cancer research, developmental biology, and other areas of the life sciences.

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### **Product from Roche Applied Science used to generate these results:**

FastStart High Fidelity PCR System.



*For information on ordering these products, see the Appendix.*



## 8.6 Analysis of DNA Methylation Patterns at the *BRCA1* CpG Island

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### Introduction

Germ-line alterations of the *BRCA1* gene confer a lifetime risk of 40% for ovarian cancers and 40-80% for breast cancers. It is likely that *BRCA1* acts as a tumor suppressor gene. *BRCA1* involvement in breast cancers does not seem to be restricted to familial cancers. Despite the absence of somatic mutation in the breast tissues, a down regulation of *BRCA1* expression is associated with malignancy in human sporadic breast cancers (Narod and Foulkes, 2004).

In tumor cells, aberrant methylation of CpG dinucleotides at the 5' end of tumor suppressor genes is frequently associated with gene silencing. However, analysis of DNA methylation patterns indicated that only a minor fraction (10-20%) of breast tumors exhibited methylated CpGs at the promoter region, position -258 to +43 from the transcription start site of *BRCA1* (Magdinier et al., 1998). Taken together these data suggest that additional epigenetic events might be involved in the down regulation of *BRCA1* in breast cancers.

The *BRCA1* gene spans 81 kb of genomic DNA and shares with *NBR2* (Next to *BRCA1* gene 2) a bidirectional promoter (Figure 8.6.1). This regulatory region is embedded in a large CpG rich region of ~2.8 kb in length, from nt -1810 to nt +974 (see Figure 8.6.1). Since DNA methylation can repress gene transcription at a distance (1-2 kb) from the promoter region, we have investigated the methylation status of this *BRCA1* CpG island.

### Materials and Methods

All enzymes were from Roche Applied Science, with the exception of *HphI* (NEN Biolabs).

### DNA Extraction

DNA was extracted from frozen pulverized tissue samples and cells with a standard proteinase K/phenol/chloroform procedure. A similar method, but with the addition of 0.001% (v/v)  $\beta$ -2 mercaptoethanol, was used to prepare decondensed DNA from spermatozoa. Human oocytes that failed to fertilize 24 hours after conventional IVF were collected from the Assisted Conception Unit (E. Herriot Hospital, Lyon, France). When DNA was analyzed from oocytes (6-10/assay), 2  $\mu$ g of pGEM-T plasmid (Promega) was added as carrier to the samples, and the samples were adjusted to a final volume of 100  $\mu$ l with 50 mM Tris-50 mM EDTA buffer that contained 0.25% SDS and 14  $\mu$ g/ml proteinase K. The mixture was incubated at 55°C for 2 hours, then the samples were processed as described in the "bisulfite modification" section below.



### PCR Based Methylation Assay

DNA extracted from tissue samples and cell lines were digested with a 5-fold excess of restriction enzyme and incubated overnight at 37°C in the appropriate buffer (Roche Applied Science). Enzymes were inactivated by heating (65°C for 1 h) and an aliquot (10 ng) of the reaction was used for PCR amplification. Besides template, each PCR sample contained standard Taq DNA Polymerase buffer (Roche Applied Science), 4% DMSO, 3 mM MgCl<sub>2</sub>, 100 μM each of the 4 dNTPs (Roche Applied Science), 0.25 μM of the primers (forward: 5' TTG GGA GGG GGC TCG GGC AT 3'; reverse: 5' CAG AGC TGG CAG CGG ACG GT 3'), and 0.6 units of Taq DNA Polymerase. DNAs were amplified by 35 cycles of PCR in an Eppendorf thermal cycler. Cycling conditions were: 1 minute denaturation at 94°C, 2 minutes annealing at 55°C, and 3 minutes extension at 72°C.

### Bisulfite Modification and Amplification

The sodium bisulfite reaction was performed on either 4 μg of DNA (3 μg of carrier DNA and 1 μg of human genomic DNA) or 2 μg of carrier plus oocyte DNA extract. Alkali-denatured DNA (0.5 M NaOH, 30 min at 37°C) was incubated for 16 h at 50°C in a total reaction volume of 100 μl that contained 3 M NaHSO<sub>3</sub> and 5 mM hydroquinone. Modified DNA was purified with the Wizard DNA Clean-up System (Promega) and eluted into 50 μl of sterile water. Modification was completed by addition of 0.3 M NaOH. DNA was precipitated with ethanol in 0.5 M ammonium acetate (pH 4.6) and resuspended in water.

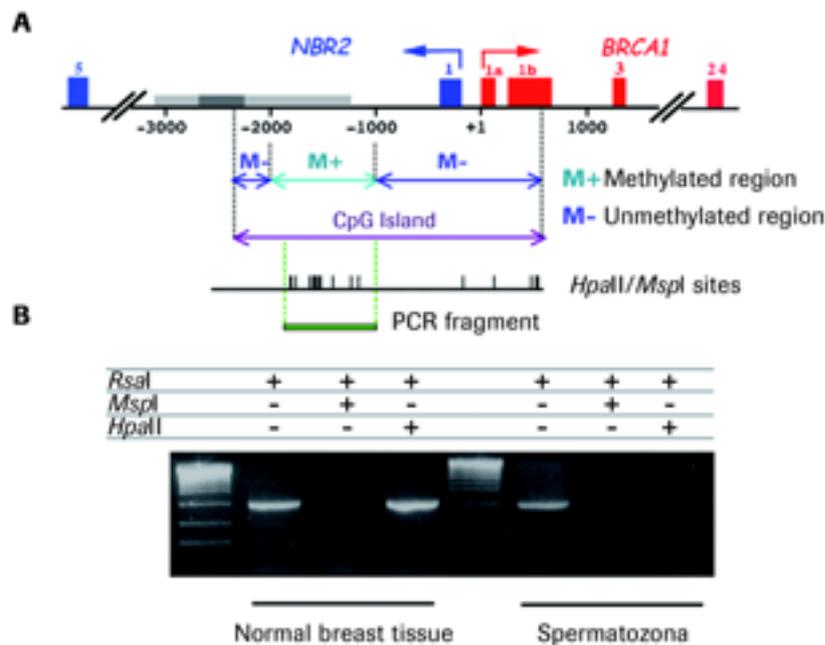
Modified DNA was amplified with a nested PCR performed in an Eppendorf thermal cycler. For the first round of PCR, each PCR sample (total volume, 100 μl) contained DNA, standard Taq DNA Polymerase buffer (Roche Applied Science), 100 μM each of the 4 dNTPs (Roche Applied Science), 3 mM MgCl<sub>2</sub>, 0.25 μM of the primers (forward: 5' TTT TGT TTT GTG TAG GGC GGT T 3'; reverse: 5' CCT TAA CGT CCA TTC TAA CCG T 3'), and 0.6 units of Taq DNA Polymerase. Cycling conditions for the first round (35 cycles) were: 1 minute denaturation at 94°C, 2 minutes annealing at 55°C, and 3 minutes extension at 72°C. An aliquot of the first amplification was reamplified with internal primers (forward: 5' TGA GAA TTT AAG TGG GGT GT 3'; reverse: 5' AAC CCT TCA ACC CAC CAC TAC 3') under the same reaction conditions.

## Result and Discussion

### PCR Based Methylation Assay

In initial experiments, we investigated the global methylation level of the BRCA1 CpG island using a PCR-based methylation assay. In order to normalize the length of genomic DNA fragments, DNA was cleaved with the *RsaI* enzyme. Then, samples were digested with *CfoI* (GCGC site) and *HpaII* (CCGG site), enzymes that are inhibited by the methylation of the internal cytosine, and as a control, with *MspI* (CCGG site), an enzyme that is insensitive to the methylation of this cytosine. *RsaI* digestion cuts within the BRCA1 CpG island fragment (nt -3053 to nt -649) and the -1714 to -1005 region was amplified by PCR. In each experiment, the sample digested with *RsaI* was also amplified to verify the efficiency of the amplification. The sequence analyzed contains 9 *HpaII* sites and 9 *CfoI* sites. PCR amplification occurs only when these sites are methylated and, therefore, uncut by the two methylation-sensitive enzymes. Representative experiments are shown in Figure 8.6.1, panel B.





**Figure 8.6.1. The *BRCA1-NBR2* locus.** (A) Transcription start sites of both genes are indicated with arrows. **red areas**, *BRCA1* exon; **blue areas**, *NBR2* exons. The locus includes a CpG island of 2784 bp in length (% G+C, 57; ObsCpG/ExpCpG, 0.65; CpGProD software, <http://pbil.univ-lyon1.fr>). **Light gray areas**, LTRc12 retro-element; **dark grey area**, AluY sequence (Repeat Masker software, version 2002).

(B) Global methylation level of the *BRCA1* CpG island. **Green bar**, PCR fragment amplified from genomic DNA digested with *RsaI* and *HpaII* or *MspI*. As expected, no PCR product was obtained from genomic DNA cleaved with the methylation-insensitive enzyme *MspI*. In contrast, after digestion of DNA from somatic cells (normal breast tissue) with the methylation-sensitive enzyme *HpaII*, PCR products were obtained, indicating that genomic DNA is methylated at the CCGG sites in the region analyzed. In contrast, no PCR product was obtained from sperm genomic DNA, indicating that these sites were unmethylated. DNA digested with *RsaI* was amplified as a control.

Data obtained indicated that *HpaII* and *CfoI* sites were methylated in human somatic tissues (including normal and tumoral somatic tissues and fetal tissues, as shown by analysis of 65 samples) and cell lines. However, human sperm DNA was found to be unmethylated (Figure 8.6.1, panel B).

This assay allows very rapid screening of the methylation status of a genomic DNA region and, by using different set of primers, a “walk” along the sequence of interest.

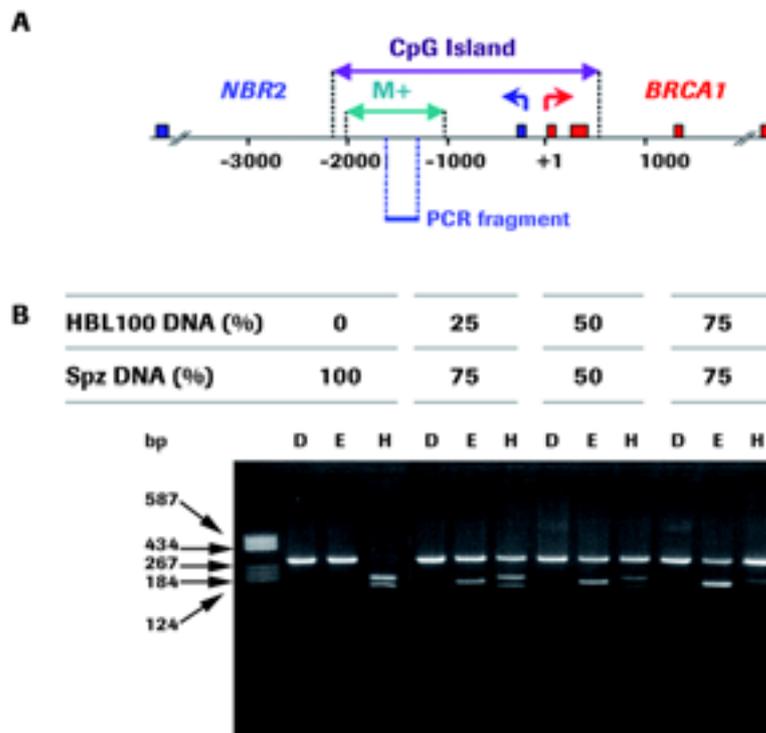
However, this assay is qualitative rather than quantitative, and in some cases methylation patterns need to be further analyzed with Southern blot experiments or by bisulfite modification of the genomic DNA to directly determine the methylation status of individual CpGs (Magdinier *et al.*, 2000).

### Validation of the Bisulfite Method

Sodium bisulfite modification followed by the sequencing of PCR products was used to determine the CpG methylation pattern. Sodium bisulfite converts unmethylated cytosines to uracils, while methylated cytosines remain unmodified. In the resultant modified DNA, uracils are replicated as thymines during PCR amplification (Martin *et al.*, 1995).

After modification, DNA was amplified with a two-step PCR. The PCR products (position -1643 to -1358) were digested with specific restriction endonucleases to determine the global methylation status of the samples. Completeness of the modification was monitored by digestion with *DdeI*, an enzyme that cleaves only unconverted DNA. PCR products obtained from methylated molecules exhibit a new *EcoRI* site at position 138, while unmethylated molecules exhibit a new *HphI* site at position 165.

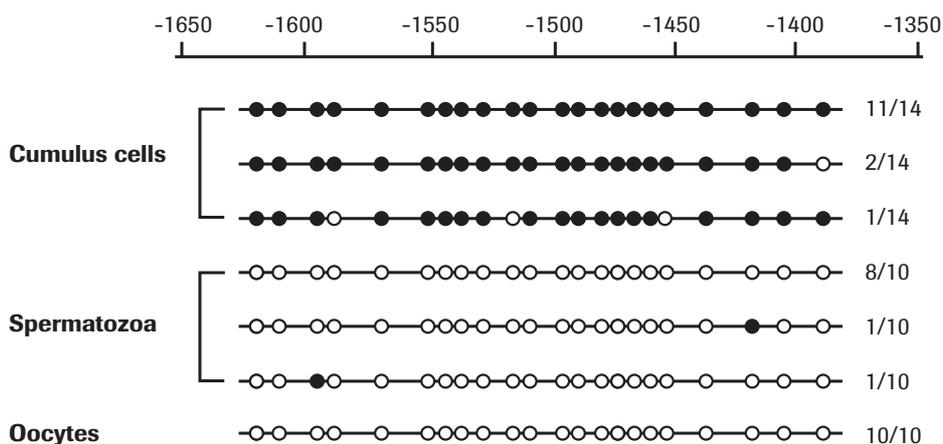
The sensitivity of PCR amplification after bisulfite modification was monitored by mixing different proportions of unmethylated DNA from spermatozoa and methylated DNA from HBL 100. For each assay, an aliquot of the PCR product was incubated with *DdeI* (unmodified DNA); *EcoRI* (methylated DNA) or *HphI* (unmethylated DNA). The results indicate that the amount of PCR product cleaved by enzymatic digestion is directly related to the ratio of methylated/unmethylated DNA used in the bisulfite modification assay (Figure 8.6.2).



**Figure 8.6.2. Validation of the bisulfite method.** (A) Legend is the same as in Figure 8.6.1, panel A. (B) Enzymatic digestion of PCR products. Genomic DNA from HBL 100 cells (methylated) and sperm DNA (unmethylated) was mixed in various proportions and modified with the bisulfite method. Then a DNA segment (position -1643 to -1358) was amplified. PCR products were digested with: *DdeI*, which cuts unmodified DNA, *EcoRI*, which cuts PCR products from methylated DNA, or *HphI*, which cuts PCR products from unmethylated DNA.

### Analysis by Bisulfite Sequencing of *BRCA1* CpG Island

DNAs from somatic tissues and gametes were modified using the bisulfite method and PCR products were cloned and sequenced. Within the region analyzed, -1643 to -1358, the 22 CpG sites analyzed were unmethylated in DNA from human oocytes and spermatozoa (Figure 8.6.3). In contrast, these CpGs were methylated in all somatic tissues and cell lines, including the somatic cells of the corona radiata surrounding the oocytes (Figure 8.6.3). The absence of DNA methylation within the CpG island in human gametes did not extend to the body of the *BRCA1* gene, since control experiments indicated that two regions of exon 11 are methylated both in somatic tissues and gametes (data not shown), suggesting that the methylation of the CpG island might play a regulatory role in *BRCA1* expression.



**Figure 8.6.3. Methylation patterns of the *BRCA1* CpG island.** After bisulfite modification and PCR amplification of the region of interest, PCR products were cloned and sequenced; for each product, 10 to 14 clones were analyzed. **filled circles**, methylated CpG; **open circles**, unmethylated CpG.

### Conclusion

Bisulfite modification of genomic DNA combined with PCR amplification of the region of interest is an inexpensive method and does not require specialized equipment. The global DNA methylation pattern of a given region can very easily be determined by enzymatic digestion of the PCR products. In addition, a more precise map of methylated regions can be determined by cloning and sequencing the PCR products.

### Acknowledgements

Parts of the figures of this publication were reprinted with permission from F. Magdinier *et al.*, FASEB J. 14; 1585-1594 (2000).

### Products from Roche Applied Science used to generate these results:

Restriction Endonucleases *CfoI*, *DdeI*, *EcoRI*, *HpaII*, *MspI*, and *RsaI*; Taq DNA Polymerase; PCR Grade dNTPs.



For information on ordering these products, see the Appendix.



## 8.7 Comparison of Several Hot-Start Taq DNA Polymerases for Detection of Differentially Expressed Genes by GeneFishing

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### Introduction

GeneFishing (Seegene) is a new proprietary method for detecting differentially expressed genes in two or more related samples. This two-step reverse transcription (RT)-PCR method modified from differential display PCR uses arbitrary primer pairs (annealing control primers, ACPs) at the PCR stage with a constant reverse primer (anchor ACP-T), which is also employed to prime the RT reaction. These ACPs have arbitrary 3'-end structures and constant 5'-end structures with an annealing regulatory region in between. With careful adjustment of cycling conditions, it is possible to amplify only those cDNAs that bear sequences complementary to the variable 3' ends of the forward primers and occur within a 5'-end region of approximately 2,000 nucleotides.

The method is simple and straightforward to perform, but it would benefit from the use of a hot-start Taq DNA Polymerase to prevent unwanted DNA synthesis during the reaction set-up, especially when multiple samples are analyzed. Therefore, we compared the suitability of several commercially available hot-start Taq DNA Polymerases for this purpose.

### Materials and Methods

C1 and C2 cDNAs originating from gestation day 4.5 and 18.5 mouse fetuses, respectively, were used as templates (supplied in the GeneFishing kit). Four different hot-start enzymes were tested with the primer pair ACP-A1 and ACP-T. The reaction mixtures for PCR were prepared according to the manufacturer's recommendations using each enzyme's own buffer (Table 8.7.1). The reaction components were assembled on ice, then the tubes were transferred to a TGradient (Biometra) thermal cycler. To activate the Taq DNA Polymerases, the tubes were incubated at 95°C for either 5 minutes (in the case of hot-start enzyme from supplier A and FastStart from Roche Applied Science) or 15 minutes (in the case of hot-start enzymes from suppliers B and C). This initial denaturation/activation phase was followed by primer annealing at 50°C for 3 minutes and second-strand synthesis at 72°C for 40 seconds. Then, a total of 40 cycles were run with the following program: 40 seconds at 94°C, 40 seconds at 65°C, and 40 seconds at 72°C. A final 5 minute elongation at 72°C completed the cycling program.

Polymerase	Supplier A	FastStart	Supplier B	Supplier C
dH <sub>2</sub> O	28.5	34.6	33.75	30.75
10 × buffer	5	5 (with Mg)	5	5(HP buffer)
25 mM MgCl <sub>2</sub>	5	1	2	5
10 mM dNTP	1	1	1	1
ACP-A1 primer	2	2	2	2
ACP-T primer	1	1	1	1
cDNA*	5	5	5	5
Taq DNA Polymerase	2.5	0.4	0.25	0.25
total µl	50	50	50	50

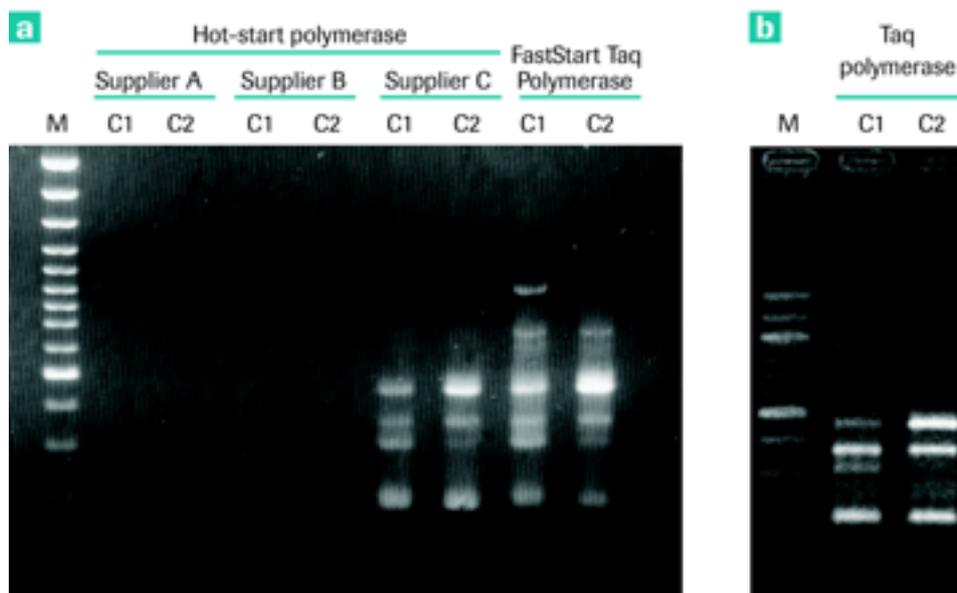
\*approximately 8 ng/µl

Table 8.7.1: PCR reaction mixtures (µl).



## Results and Discussion

The hot-start Taq DNA Polymerases from suppliers A and B failed to amplify any product from the two cDNAs (Figure 8.7.1). The hot-start Taq DNA Polymerase from supplier C generated the smaller products (up to 500 bp), but only FastStart Taq DNA Polymerase was able to produce all the expected bands including the largest two (approximately 950 bp and 1,100 bp).

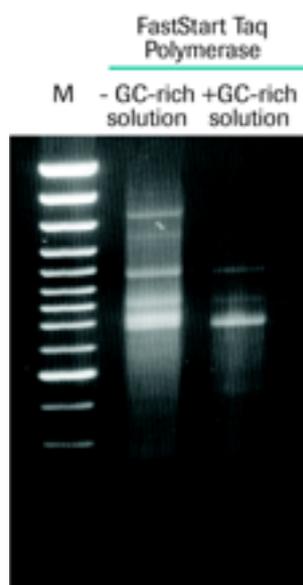


**Figure 8.7.1. (a) Gel electrophoresis of PCR products** generated with ACP-A1/ACP-T primers and with one of the indicated hot-start polymerases (from suppliers A, B, C, or FastStart Taq DNA Polymerase). The cDNA template was either C1 or C2 (M, 100 bp marker). **(b)** The pattern of bands obtained with Taq DNA Polymerase from C1 and C2 cDNA templates with the same primer pair (M, 100 bp marker, Seegene).

The degree of DNA polymerase activity is critical during the first cycle of the GeneFishing procedure, as second-strand synthesis is in process during this phase. All the remaining cycles have a relatively high annealing temperature. No product is generated if the first cycle is unsuccessful – in this case the arbitrary forward primer cannot anneal. Although all the hot-start enzymes tested were activated for the recommended periods at an appropriate temperature, the enzymes that failed may not have reached sufficient activity during this activation step. Alternatively, the buffer compositions of these enzymes did not favor annealing at the temperatures used.

Subsequent fine-tuning of the cycling conditions for FastStart Taq DNA Polymerase showed that even for this enzyme, it was advantageous to increase the initial denaturation time from 5 minutes to 8 minutes in order to elevate its activity for second-strand synthesis. Furthermore, the yield of long PCR products (up to 2 kb) improved when we increased the elongation time to 2 minutes for the first cycle, to 45 seconds for the next 15 cycles, and then stepwise by 1 second per cycle for the remaining 25 cycles.

FastStart Taq DNA Polymerase is supplied with an optional solution that is supposed to enhance amplification of GC-rich templates. The effect of this solution was tested, with another primer pair (ACP-A6/ACP-T), on a rat liver cDNA sample. For the present experimental conditions, a reaction mixture without the GC-RICH Resolution Solution proved to perform better (Figure 8.7.2).



**Figure 8.7.2. Banding patterns** generated with FastStart Taq DNA Polymerase and ACP-A6/ACP-T primers from an untreated rat liver cDNA in the absence or presence of the GC-RICH Resolution Solution (M, 100 bp marker).

So far, we have used almost 30 primer pairs with FastStart Taq DNA Polymerase in the GeneFishing procedure, and the results have invariably been highly satisfactory. The hot-start modification of the original method offers flexibility and convenience during reaction set-up without compromising the outcome. Thus, a large number of individual cDNA samples can be safely processed at the same time for comparative analysis of gene expression levels.

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**Product from Roche Applied Science used to generate these results:**

FastStart Taq DNA Polymerase.



*For information on ordering these products, see the Appendix.*

## 8.8 Transcriptional Organization of the O Antigen Biosynthesis Cluster in the GC-Rich Bacterium *Burkholderia cenocepacia*

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### Introduction

Infections with members of the *Burkholderia cepacia* complex have become a serious threat for immunocompromised individuals, particularly those who suffer from cystic fibrosis and chronic granulomatous disease (Mahenthiralingam *et al.*, 2005). In Canada, *B. cenocepacia* is the *B. cepacia* complex species most commonly found in infected subjects (approximately 83% of all the isolates). The *B. cenocepacia* type strain J2315 has a genome size of 8.056 Mb organized into three chromosomes and one large plasmid ([http://www.sanger.ac.uk/Projects/B\\_cenocepacia/](http://www.sanger.ac.uk/Projects/B_cenocepacia/)).

In general, it is difficult to perform molecular investigations in *B. cenocepacia*, in part due to its high GC content (66.9%), the abundance of insertion elements in its genome, and the multiple antibiotic resistance of all strains. Recently, our laboratory reported the isolation of *B. cenocepacia* mutants which were attenuated for survival in a rat agar bead model of lung infection. Four of these mutants had transposon insertions in genes of an O antigen lipopolysaccharide (LPS) biosynthesis cluster, indicating that production of O antigen is important for bacterial survival *in vivo* (Hunt *et al.*, 2004). Further studies have shown that the O antigen cluster in *B. cenocepacia* is complex and consists of several transcriptional units (Ortega *et al.*, 2005). We investigated the transcriptional organization of this cluster by RT-PCR, using Transcriptor Reverse Transcriptase (Roche Applied Science) and primers that allowed us to detect co-transcription between genes. The Transcriptor Reverse Transcriptase allowed GC-rich RNA with a great deal of secondary structure to be optimally transcribed.

### Material and Methods

#### Total RNA isolation

The method described by Glisin *et al.* (1974) was used to isolate total RNA from the *B. cenocepacia* strain K56-2. A 25 ml culture of *B. cenocepacia* K56-2 was grown in Luria broth to an OD<sub>600</sub> of 0.6. Bacterial cells were collected by centrifugation, resuspended in 3.5 ml of RNase-free TESS buffer (20 mM Tris pH 7.6, 10 mM EDTA, 100 mM NaCl, 1% SDS), and lysed by heating (95°C for 3 minutes). Cesium chloride powder was added to the lysate at a final concentration of 1 g/ml and the lysate was layered atop a 1 ml CsCl cushion (prepared with 5.7 M CsCl, 0.1 M EDTA) in SW50.1 tubes (Beckman Coulter). The cell lysate was centrifuged at 39,000 rpm for 16 hours at 20°C. After centrifugation, a thick band containing DNA and cell debris was visible in the middle of the tubes, while the RNA was deposited as a clear pellet at the bottom of the tubes. After the tube contents were aspirated, the bottom of each tube was carefully cut with a hot scalpel and the RNA pellet was dissolved in 100 µl of 20 mM sodium acetate containing 1 mM EDTA. A volume of 200 µl of ethanol was added to the RNA and the tube was stored at -20°C. An aliquot of the precipitated RNA was centrifuged. The pellet was then washed, dried, resuspended in RNase-free water, and treated with DNase I. DNase I was subsequently eliminated in a cleanup protocol that used a commercially available RNA isolation kit. The resulting RNA was used for RT-PCR analysis.

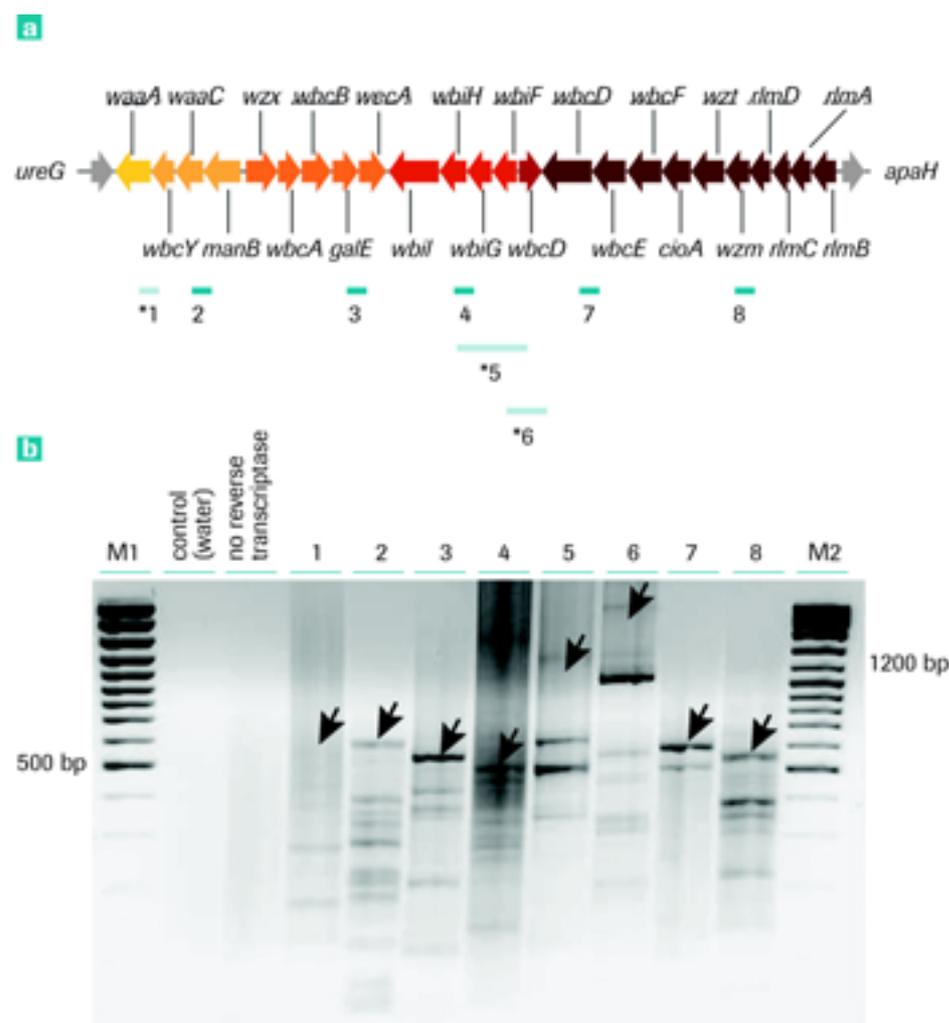


### RT-PCR analysis

Reverse transcription was performed with Transcriptor reverse transcriptase and primers that spanned intergenic regions. The sequence of the primers can be obtained from the authors upon request. To ensure absence of any RNase activity, Protector RNase Inhibitor (Roche Applied Science) was used in all the reverse transcription reactions. The resulting cDNA was subjected to PCR with Taq DNA Polymerase. The conditions for the amplification were: 1 × 2 minutes at 94°C, followed by 10 cycles × (94°C/10 seconds, 54°C/30 seconds, 72°C/2 minutes), followed by 30 cycles × (94°C/10 seconds, 59°C/30 seconds, 72°C/2 minutes), and a final extension of 1 × 7 minutes at 72°C. The PCR products were visualized on a 1.7% (wt/vol) agarose gel.

### Results and Discussion

Bacterial mRNAs are usually very unstable due to their short half lives. The cesium chloride-based method was used to obtain large quantities of RNA, but also because it rapidly inactivates RNases during the lysis step. From a 25 ml culture, RNA was purified at a concentration of 14.2 mg/ml. The RNA was stored in ethanol at -20°C. This allowed us to perform all the RT-PCR analysis with the same RNA preparation since it can be kept for a long time, thus ensuring uniformity of the results from the various RT-PCR analyses.



**Figure 8.8.1. Analysis of the transcriptional organization of the O antigen cluster by RT-PCR.** (a) Genetic map of the O antigen cluster of *B. cenocepacia* K56-2. The flanking genes are represented by grey arrows. All the genes from the O antigen cluster that are transcribed as part of the same unit are depicted with the same color.

Accordingly, six transcriptional units were found. The bars below the map indicate the boundaries of the expected amplification products. Those indicated with asterisks denote fragments that were not amplified, and thus correspond to regions that were not cotranscribed. The numbers indicate the corresponding lanes in panel b. **(b)** Agarose gel containing appropriate controls and amplification reactions from the eight regions selected for these experiments. The arrows indicate the presence or absence of the expected amplification products.

For each PCR, the appropriate controls (with water and with cDNA synthesized in the absence of reverse transcriptase) were included to ensure that the amplifications obtained were a result of cDNA and not of contaminating genomic DNA. Figure 8.8.1 clearly shows that after DNase treatment, no amplification was obtained in the water control or in the reaction without reverse transcriptase. This confirms that any products obtained from the subsequent PCRs were due to the presence of the corresponding cDNA, and not from chromosomal DNA contamination. RT-PCR analysis of the O antigen synthesis cluster showed amplification in each case in which neighboring genes were cotranscribed across their boundaries. This occurred for five out of the eight regions that were studied. Cotranscription was detected in regions 2, 3, 4, 7 and 8, while regions 1, 5, and 6 did not give amplification for the expected fragments (Figure 8.8.1). Based on these results we propose the following transcriptional units: *rmlBACD-wzm-wzt-vioA-wbcFED*, *wbcC*, *wbiFGHI*, *wzx-wbcAB-galE-wecA*, *manB-waaC-wbcY*, *waaA*. The elucidation of the transcriptional organization of the O antigen cluster in *B. cenocepacia* K56-2 provides valuable information that will help us understand how O antigens are synthesized in *B. cenocepacia*.

Neither the high GC content of the *B. cenocepacia* genome (66.9%) nor RNase contamination caused any problems. Transcriptor Reverse Transcriptase transcribes very efficiently even when templates have high GC content – this enzyme can efficiently use RNA templates with up to 70% GC content. Moreover, use of the Protector RNase Inhibitor at every step of the RT-PCR improved the quality of the templates.

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#### **Products from Roche Applied Science used to generate these results:**

Transcriptor First Strand cDNA Synthesis Kit, Transcriptor Reverse Transcriptase, Protector RNase Inhibitor.



For information on ordering these products, see the Appendix.



## 8.9 Transcription Analysis of a Retroviral Vector System that Transfers Intron-Containing Genes

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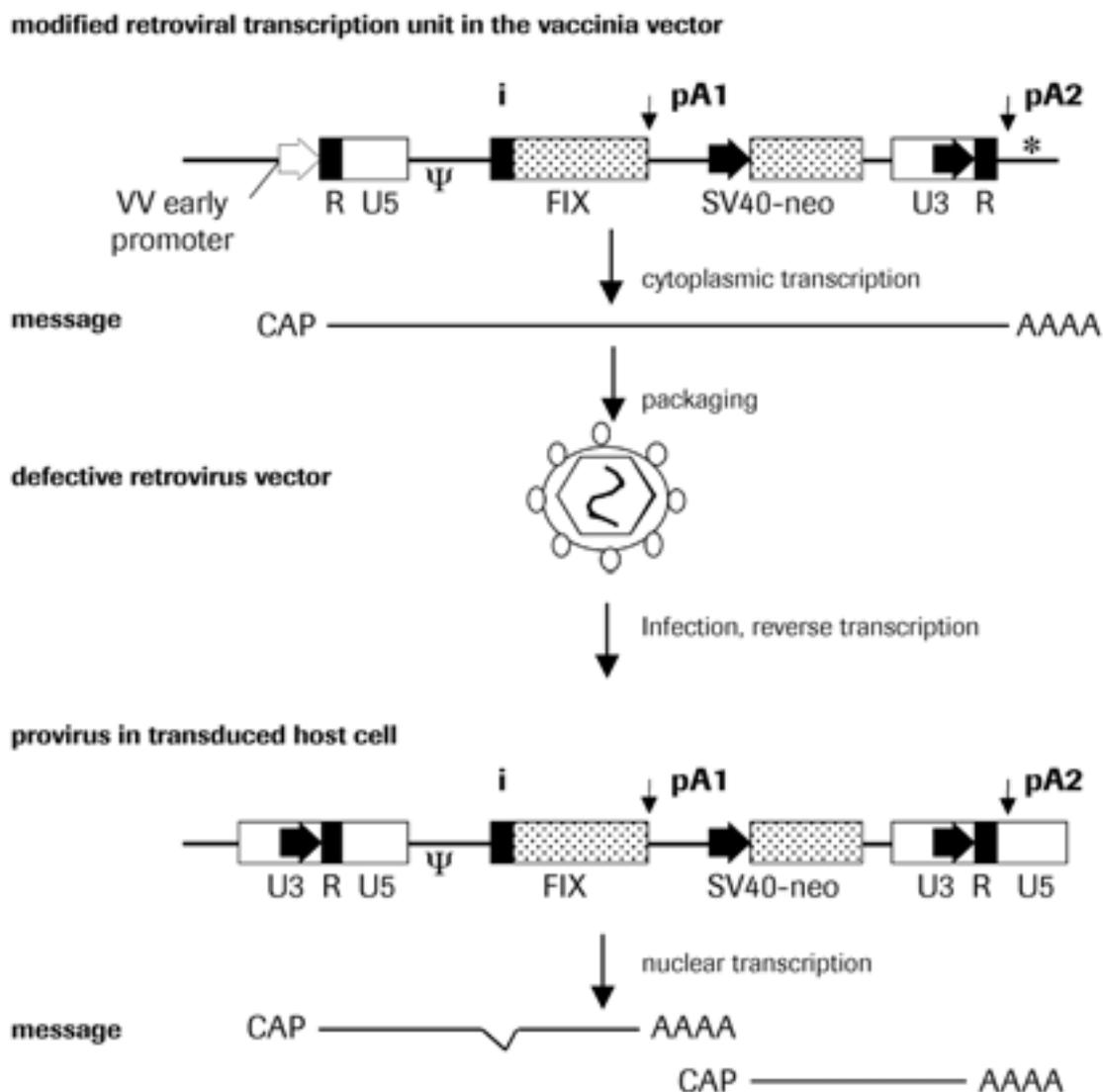
\* Corresponding author: holzerg@baxter.com

### Introduction

Introns and polyadenylation (pA) sites are known to improve transcript stability and nuclear-cytoplasmic transport (Brinster *et al.*, 1988) and are normally present in efficient gene expression vectors. With standard retroviral vectors, the nature of replication via RNA transcripts (Coffin, 1996) precludes the transfer of intron-containing genes. To overcome this shortcoming, we explored retrovirus-mediated transfer of gene cassettes that contain RNA processing signals (*e.g.*, splice introns or polyadenylation sites). The vehicle for this transfer was a vector system that contained a combination of vaccinia virus and retrovirus.

Briefly, the hybrid vaccinia - retrovirus vector system (Holzer *et al.*, 1999; Konetschny *et al.*, 2003) uses vaccinia virus to express all components of the retroviral vector particle. The particles obtained display the properties of normal retroviral vectors, such as stable integration into the host cell and transgene expression. Production of the retroviral vectors following vaccinia virus infection occurs strictly in the cytoplasm, and nuclear RNA processing signals have no impact on transcript modifications. Here we describe the PCR analysis of transcriptional events involving a hybrid retroviral vector (Figure 8.9.1) that contains an intron and a custom polyadenylation signal (Konetschny *et al.*, 2002).





**Figure 8.9.1. Schematic representation of the genetic elements in the viral genomes and transduced cells.** The vaccinia virus (VV) vector contains a modified retroviral transcription unit. A vaccinia virus promoter (white arrow) is fused to the repeat region (R) of the LTR and drives the transcription of the full length message, terminating at the vaccinia transcriptional stop signal (asterisk). Retroviral vector (RNA) genomes, which are produced and packaged upon vaccinia virus infection, retain the intron and the internal polyA (pA1) site that are engineered into the vector. During reverse transcription, the LTR promoter (located in the U3 sequence, bold arrow) is reconstituted. In the retrovirally transduced host cell, the nuclear transcription signals such as promoters, a small SV40 intron (i), and two pA sites (pA1 and pA2) define two separate transcriptional units, one containing the foreign gene (FIX) and one containing the selection marker (neo).

RT-PCR was used to monitor the splicing events throughout the experiment, and also to estimate splicing efficiency. For this purpose, mRNAs were reverse transcribed to cDNA, and the genetic structure of the primary vaccinia DNA, of the retroviral vector RNA, and finally of the genomic DNA of the transduced cell clones was analyzed. Primers were designed to give short amplicons that differ in length depending on the splicing of the template. Also, utilization of the polyadenylation signal was demonstrated with a combination of RT-PCR and sequencing.

## Materials and Methods

### RT-PCR Analysis of Foreign Gene Transcription and Splicing

We used an oligo(dT) primer for reverse transcription of total RNA into cDNA. From 500 ng of RNA, first-strand cDNA was synthesized with reverse transcriptase at 42°C for 45 minutes. RNA/DNA hybrids were denatured and the reverse transcriptase was inactivated at 95°C for 5 minutes before the cDNA was used as template in the PCR reactions.

To visualize splicing of transcripts, PCR was done with a primer set that frames the splice intron to give clearly discernable PCR products in the presence or absence of the intron. Amplification was performed with Taq DNA Polymerase (Roche Applied Science). The thermal cycling protocol (for 35 cycles of PCR) was: 45 seconds denaturation at 95°C, 45 seconds annealing at 59°C, and 90 seconds extension at 72°C. All primers are described in Konetschny *et al.* (2002).

### Analysis of Transcriptional Termination at the Internal pA Site

The same cDNA described above was also used as PCR template with a different primer pair. In this reaction, the forward primer binds to the gene of interest upstream from the internal pA site, and the reverse primer binds to the tag at the end of the cDNA strand (Figure 8.9.3, panel A). Amplification was performed with Taq DNA Polymerase (Roche Applied Science). The thermal cycling protocol (for 35 cycles of PCR) was: 20 seconds denaturation at 95°C, 30 seconds annealing at 54°C, and 70 seconds extension at 72°C. The PCR products were purified with a spin column protocol and sequenced.

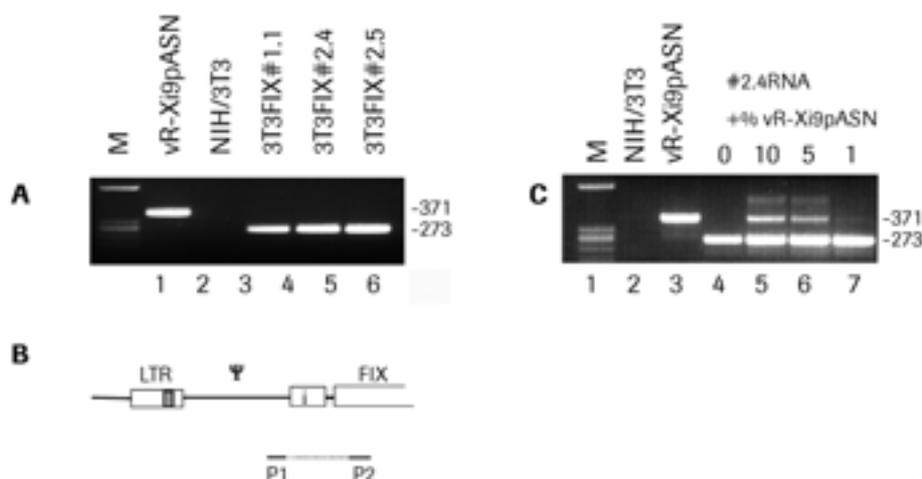
## Results and Discussion

To investigate the role of transcription signals in a complex virus vector system, we designed RT-PCR experiments to track splicing and polyadenylation of the transcripts.

### Transcription and Splicing

For monitoring the presence of the splice intron at different stages, a primer pair was designed that frames the SV40 intron (Figure 8.9.2). A larger amplicon of 371 bp arises from the unspliced RNA. This signal was observed when the template was derived from vector-infected cells in which no splicing of the message takes place. When RNA from the transduced cell clones was used, a single, smaller amplicon (273 bp) was obtained, indicating correct splicing of the message. Splicing occurred quantitatively, as indicated by the absence of a 371 bp band.





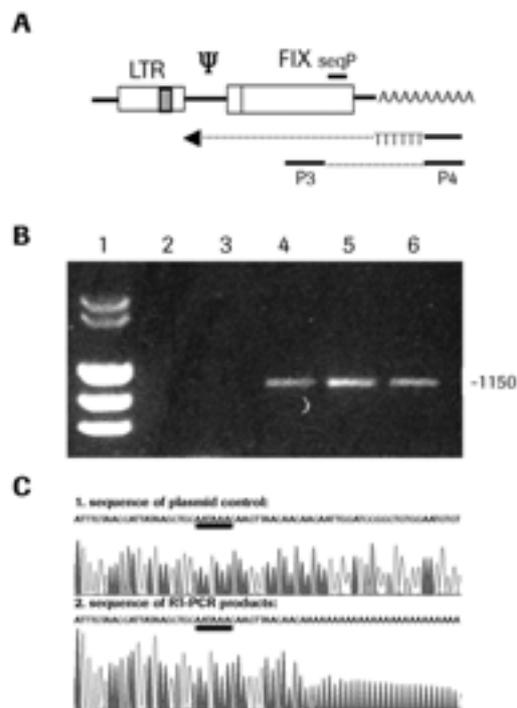
**Figure 8.9.2. (A) RT-PCR analysis of transcripts from the vaccinia virus-infected cells and the transduced cell clones. Lane 1, size markers (M); Lane 2, RNA of cells infected with the recombinant vaccinia virus; Lane 3, negative control RNA of NIH 3T3 cells; lanes 4 to 6, RNA of three transduced clones. (B) Schematic diagram of the primers (P1-P2) and the template used. (C) Transcripts of a transduced cell clone spiked with unspliced vaccinia virus RNA. Lane 1, marker (M); Lane 2, RNA of the NIH 3T3 negative control; Lane 3, RNA of cells infected with the recombinant vaccinia virus; Lanes 4 to 7, RNA of a transduced cell clone spiked with the indicated amounts of unspliced viral RNA. The numbers on the right are the sizes of the bands in base pairs.**

In order to estimate the detection limit for potentially unspliced RNA in the transduced clones, mixing experiments were performed. Increasing amounts of unspliced virus RNA derived from vaccinia vector-infected cells were spiked into the RNA of one cell clone, which was subsequently analyzed by RT-PCR as described above. Addition of as little as 1% cDNA from unspliced RNA resulted in the appearance of an additional band in the 371-bp size range (Figure 8.9.2, panel C), indicating that at least 99% of the RNA in the cell clones was spliced.

Reliable discrimination of two different template species clearly depends on the PCR settings chosen. In our experiment, the distance between the primers was very short (max. 371 bp), and the size difference between the larger and the smaller PCR product (from spliced RNA) was only 100 bp. At these settings, the simultaneous amplification of two related fragments was not limited by the elongation rate or the frequently observed preference of the reaction for a shorter PCR product.

### Termination and Polyadenylation

To analyze termination, messenger RNA was first subjected to Northern blotting. The blot results [not shown; for details see Konetschny *et al.* (2002)] demonstrated that the major part of the message terminated at the expected region. The transcripts were further analyzed by RT-PCR and subsequent sequencing of the polyadenylated 3'-region. As described above, the cDNAs were synthesized in an RT reaction using a tagged oligo(dT) primer. The amplification step was performed with one primer that was complementary to the tag introduced by the oligo(dT) primer, and another primer that was specific for the gene of interest. When RNA from the transduced cell clones was used as template, an amplicon of about 1,150 bp was obtained. These results indicate that the transcript had been terminated and polyadenylated at the pA site that was introduced downstream from the gene of interest (Figure 8.9.3).



**Figure 8.9.3. RT-PCR of transcripts terminating at the internal pA site and sequence analysis of cDNAs.** (A) Schematic diagram of the primers and the template used to map pA. (B) Agarose gel electrophoresis showing the results of the PCR analysis. Lane 1, size markers; lane 2, RNA of cells infected with the hybrid virus vR-Xi9pASN; lane 3, negative control RNA of NIH 3T3 cells; lanes 4 to 6, RNA of the transduced clones. The number on the right is the size of the bands (in base pairs). (C) Sequence of plasmid control and cDNAs derived from the internally terminated transcripts.

Sequence analysis of these amplicons, produced with a primer that binds about 250 bp upstream from the 3' end of the foreign gene, allowed us to define the 3' end of the message in the cell clones and confirm that the pA signal functioned correctly. It should be noted that a single 1,150 bp RT-PCR product was obtained; no larger amplicon was seen in the reaction, even though the template contained low quantities of run-through mRNA, which were detectable by Northern blotting. Since only one product was present, we could sequence it directly without having to first isolate it.

A control reaction was performed with mRNA from the vaccinia infection, which contains only run-through transcripts that should terminate about 1.8 kb further downstream, *i.e.* at the VV transcription stop signal located at the end of the retroviral 3'-LTR (see Figure 8.9.1). Even in this control reaction, the large amplicon (theoretical size, 3 kb) was not obtained. The use of limiting PCR conditions (extension time only 70 seconds) prevented amplification of this 3 kb run-through transcript, hence allowing the PCR to generate a specific, sequencing-grade product from the 1.15 kb transcript of interest.



### **Summary**

In summary, we used Taq DNA Polymerase successfully in two types of RT-PCR experiments with contrasting purposes. By choosing the appropriate extension time, we could design reactions either for simultaneous semi-quantitative detection of two mRNA species, or for specific suppression of background signals.

### **Acknowledgements**

Parts of the figures in this publication were reprinted from Konetschny *et al.* (2002) by permission of the American Society of Microbiology.

### **Product from Roche Applied Science used to generate these results:**

Taq DNA Polymerase.



*For information on ordering this product, see the Appendix.*

## 8.10 Quantification of *BRCA1* Expression Levels with Standard Roche RT-PCR Reagents: A Sensitive Method for Detecting Low Amounts of Transcripts

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### Introduction

Approximately a decade ago, the breast cancer susceptibility gene, *BRCA1*, was mapped to the long arm of chromosome 17 (17q21) by genetic linkage analysis. Mutations in the *BRCA1* locus account for the majority of families with multiple members affected with breast or ovarian cancer. Indeed, women who inherit a *BRCA1* mutation have a 40-80 % risk of developing breast cancer in their lifetime as well as a 40% risk of having ovarian cancer.

Up to 90% of breast cancers are sporadic. However, mutations in *BRCA1* seem to be rare in sporadic breast tumors.

*BRCA1* is expressed at a low level in a wide range of normal and pathological human tissues, which suggests a generic activity in cell homeostasis. The *BRCA1* tumor suppressor gene encodes a 220 kDa nuclear phosphoprotein that participates in the DNA damage response and acts to maintain the integrity of the genome. In addition to genome maintenance, *BRCA1* has been found to broadly regulate gene transcription. Nevertheless, the broad caretaker function of *BRCA1* does not explain why mutations lead to cancer only in certain organs. This organ specificity suggests the involvement of tissue-specific coregulators. Therefore, we hypothesized that dysregulation of *BRCA1* gene expression in human samples, especially sporadic breast cancer, might also account for the pathogenesis. In order to determine the putative variations in *BRCA1* expression levels, we set up a quantitative two-step reverse transcription-polymerase chain reaction (RT-PCR) based on competitive amplification between a chimerical *BRCA1* synthetic transcript and the endogenous messenger RNA.



## Materials & Methods

### Production of competitor RNA

A BRCA1 cDNA fragment (from position 390 to position 647, exons 4 to 6) was amplified by RT-PCR using C3 primers: 5' TGT GCT TTT CAG CTT GAC ACA GG 3' and 5' CGT CTT TTG AGG TTG TAT CCG CTG 3' (Friedman et al., 1994). After it was cloned into a pGEM-T vector (Promega), the fragment was cleaved at position 112 with *NotI* and a blunt-ended oligonucleotide was ligated at this site. This vector contains a promoter site for the T7 RNA Polymerase (Roche Applied Science); this polymerase was used to produce the corresponding chimerical RNA, according to instructions provided by the manufacturer. After purification, this competitor RNA was quantified by spectrophotometry, and aliquots were diluted in the presence of yeast tRNA as a carrier (Figure 8.10.1, panel A).

### Extraction of RNA and RT-PCR

RNA extraction was performed on cultured cells or human biopsies that had been snap frozen in liquid nitrogen using standard procedures (Magdinier et al., 1998, Magdinier et al., 1999). Serial dilutions of total RNA were quantified by spectrophotometry and by comparing the RNA with known amounts of standard RNA (Roche Applied Science) on agarose gels.

Two-step RT-PCR was performed in a total reaction volume of 100  $\mu$ l. Each reaction contained either 0.3 or 0.6  $\mu$ g of total RNA, varying amounts of competitor RNA, standard Taq DNA Polymerase buffer (Roche Applied Science), 3.0 mM MgCl<sub>2</sub>, 200  $\mu$ M each of the 4 dNTPs (Roche Applied Science) and C3 primers. After the reaction was denatured at 92°C for 2 min, 6 units of M-MuLV Reverse Transcriptase (Roche Applied Science) was added, and the reaction mixture was incubated for 35 min at 42°C. Reverse transcriptase was then inactivated by heating (94°C, 3 minutes). After the tube was cooled to 4°C, 0.6 unit of Taq DNA Polymerase (Roche Applied Science) was added. The cDNA product was amplified by 35 cycles of PCR in an Eppendorf thermal cycler. Cycling conditions were: 1 minute denaturation at 94°C, 2 minutes annealing at 55°C, and 3 minutes extension at 72°C. Under these conditions, heterodimers between PCR products derived from mRNA and the competitor were not detected.

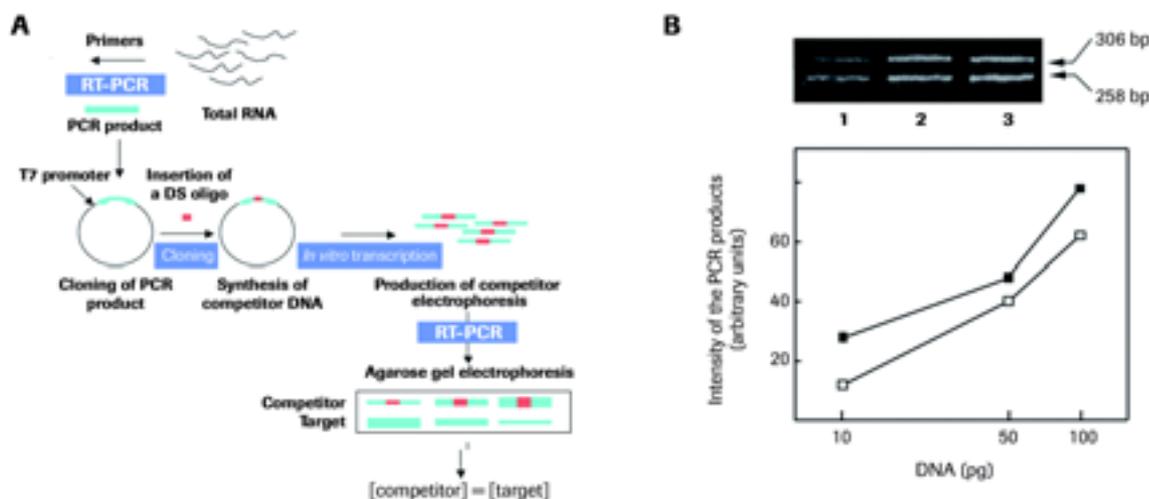
Aliquots of product were then analyzed on a 2% agarose gel containing 0.1  $\mu$ g/ml of ethidium bromide. Photographs of the gels were scanned and the intensity of the 2 PCR product bands was determined using image analyzer software (Wayne Rasband, NIH). The amounts of PCR products (corresponding to BRCA1 mRNA and to synthetic RNA) were plotted against the initial amounts of synthetic RNA added to the reaction tubes (Figures 8.10.1, panel A; 8.10.2, panel A). The abscissa below the intersection of the curves represents an estimate of the equivalence point between the initial amount of the competitor molecules and the number of copies of BRCA1 mRNA in 0.3  $\mu$ g of sample RNA.



## Results

### Validation of the Competitive RT-PCR Assay

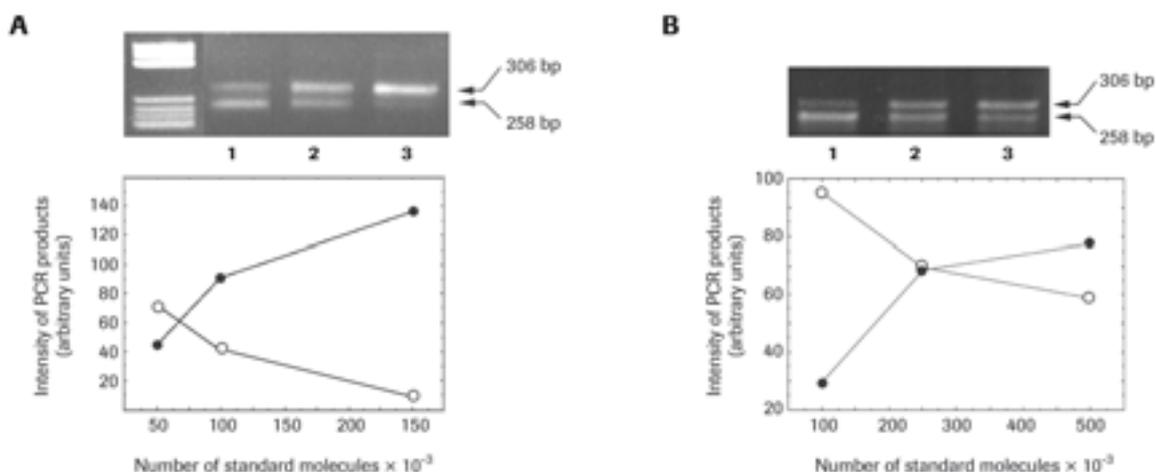
In order to evaluate the sensitivity of the RT-PCR, serial dilutions of the chimerical competitor and wild-type *BRCA1* cDNA were amplified (Figure 8.10.1, panel B). The amount of PCR product was then determined by densitometry on ethidium-bromide-stained agarose gels and plotted against the initial concentration of material. Data obtained show that the signal was proportional to the amount of cDNA added to the reaction mixture, indicating that endogenous *BRCA1* mRNA and synthetic mRNA are amplified at the same rate (Figure 8.10.1, panel B). The signal is proportional to the log of the amount of synthetic RNA over a wide range of concentrations, from  $3 \times 10^3$  to  $10^6$  copies per assay (data not shown). Furthermore, the quantification of *BRCA1* mRNA/ $\mu\text{g}$  of total RNA is not affected by the amount of total RNA analyzed (Ribieras *et al.*, 1997). No PCR by-products or primer-dimers were seen in the assays even when target amounts were very low.



**Figure 8.10.1. Design and validation of a competitive RT-PCR assay.** (A) A cDNA encompassing exon 4 to 6 of the human *BRCA1* gene was modified by integration of a double-stranded oligonucleotide. After cloning, the chimerical fragment was transcribed *in vitro* and the resulting RNA was used for a quantitative RT-PCR assay. (B) The efficiency of amplification of the synthetic RNA is similar to the rate of amplification of the endogenous wild-type *BRCA1* cDNA fragment over a range of concentrations (10 to 100 pg of cDNA).

### Quantification of *BRCA1* Expression Levels

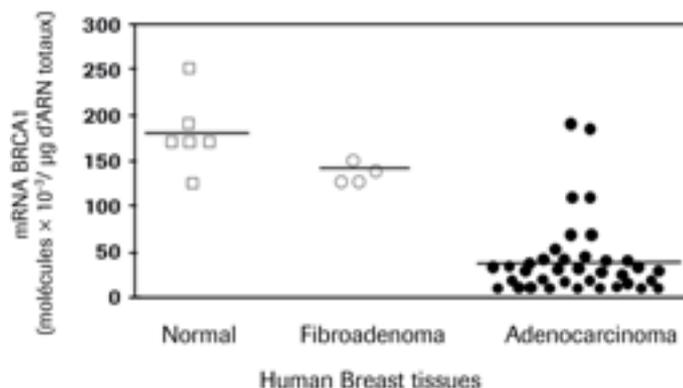
After these preliminary validations, the amount of *BRCA1* mRNA was determined in several human cell lines by competitive RT-PCR analysis of total RNA extracted from human biopsies. The assays were performed with serial dilutions of the competitor mixed with a constant amount of sample (total) RNA. The quantity of PCR products were plotted against the initial amount of synthetic RNA added to the tubes. The abscissa at the intersection of the curves represents an estimate of the equivalence point between the initial amount of the competitor molecules and the number of copies of *BRCA1* mRNA per cell, assuming that each cell contains 25  $\mu\text{g}$  of total RNA. This method allowed us to measure the level of *BRCA1* transcript over a wide range of values and was valid even for samples that contained only a few copies of *BRCA1* mRNA (Figure 8.10.2). Furthermore, the values obtained with this technique were consistent with protein levels determined on western blots (Magdinier *et al.*, 1999).



**Figure 8.10.2. Quantification of *BRCA1* expression with competitive RT-PCR.** The graphs show the intensity of the PCR product bands plotted against the initial amount of competitor RNA. Reactions contained varying amounts (serial dilutions) of chimeral *BRCA1* RNA and a fixed amount (either 0.3  $\mu\text{g}$  or 0.6  $\mu\text{g}$ ) of total RNA. Fifteen microliters of each reaction (total volume, 100  $\mu\text{l}$ ) was analyzed on agarose gels; the 306-bp band corresponds to the expected size of the chimeral PCR product, and the 258-bp band to wild-type *BRCA1* cDNA. **(A).** Quantification in samples expressing *BRCA1* at a low level. **(B).** Quantification in samples expressing *BRCA1* at a high level.

The competitive RT-PCR method was also used to investigate the steady state level of *BRCA1* mRNA in normal and pathological breast tissues. The data obtained are summarized in Figure 8.10.3 and indicate that the *BRC*

*A1* mRNA level is significantly decreased ( $p = 0.0003$ ) in malignant specimens ( $37.054 \pm 4.24 \times 10^3$  copies/ $\mu\text{g}$  of total RNA) relative to normal breast tissues ( $179.2 \pm 40.8 \times 10^3$  copies/ $\mu\text{g}$  of total RNA). In the fibroadenoma samples, the expression level of *BRCA1* was similar to the level measured in normal breast samples ( $134.7 \pm 10.44 \times 10^3$  copies/ $\mu\text{g}$  of total RNA). In conclusion, the majority of the breast cancer samples analyzed exhibit a 10- to 12-fold decrease in *BRCA1* mRNA level relative to normal breast tissues or benign lesions. However, this low level of *BRCA1* mRNA does not seem to be associated with a specific histological type of cancer. Therefore, these results suggest that the down regulation of *BRCA1* is a feature of sporadic breast cancer.



**Figure 8.10.3. Analysis of *BRCA1* gene expression in normal and pathological human breast samples.** Quantification was performed in six normal breast samples (open squares), four fibroadenomas (open circles) and 37 breast cancers (filled circles). For each group, the horizontal line corresponds to the median value of expression level.



### **Conclusion**

Although real-time quantitative RT-PCR is a very powerful tool to study the expression of genes, this technology is not always available. The technique described in this report can be set up easily in any laboratory possessing standard molecular biology equipment such as a thermal cycler, agarose gel electrophoresis equipment and a desktop image scanner. Furthermore, this technique is highly reproducible, even when applied to very low amounts of material.

### **Products from Roche Applied Science used to generate these results:**

T7 RNA Polymerase, M-MuLV Reverse Transcriptase, Taq DNA Polymerase, dNTPs, RNA Molecular Weight Markers.



*For information on ordering these products, see the Appendix.*



## 8.11 Tailor-made Solutions Exemplified with the High-throughput 5' RACE Kit

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### Introduction

Rapid amplification of 5' cDNA ends (5' RACE), a PCR-based method designed to characterize the

5' ends of mRNAs (Frohman M.A. et al., 1988), has been adapted for the identification of endogenous loci associated with insertions of gene trap vectors (Skarnes W.C. et al., 1992). Direct sequencing of 5' RACE products (Townley D.J. et al., 1997) has been used by several large-scale gene trapping efforts to generate a library of insertional mutations in mouse embryonic stem (ES) cells (see BayGenomics). In close collaboration with scientists at Roche Diagnostics, we have optimized 5' RACE and developed a kit for high-throughput characterization of gene trap ES cell lines. The tailor-made HTS 5' RACE kit provides a significant improvement in the quality and length of sequence tags generated by 5' RACE, and consequently, more of the sequences can be annotated to the mouse genome.

With the completion of the human and mouse genome sequences, attention is now focusing on the functional analysis of the ~25,000 genes in the mammalian genome (Auwerx J. et al., 2004; Austin C.P. et al., 2004). Gene trapping in mouse embryonic stem (ES) cells provides a cost-effective approach to creating insertional mutations in a substantial fraction of genes in mice (Skarnes W.C. et al., 2004).

Gene trap vectors contain a splice acceptor site upstream of a promoterless bgeo (b-galactosidase/ neomycin phosphotransferase fusion) reporter gene that is activated following insertions in genes expressed in ES cells. To identify the endogenous gene associated with each gene trap insertion, 5' RACE is used to amplify the cDNA sequence upstream of the bgeo reporter gene followed by automated fluorescent sequencing to generate a short sequence tag for each insertion event (Townley D.J. et al., 1997). Application of this method on a high-throughput scale proved to be problematic.

Here we describe the results of a collaboration with Roche Diagnostics to develop a custom highthroughput 5' RACE kit. This Roche HTS 5' RACE kit simplifies the protocol and dramatically improves the length and quality of sequence tags generated from gene trap cell lines.



### Materials and Methods

First, 100 µg of linearized pGT0lxr vector was electroporated into 10<sup>8</sup> ES cells and selected in 200 µg/ml G418 for up to 9 days. Colonies containing random gene trap insertions were picked into 48-well tissue culture plates and when confluent, re-arrayed into 24-well tissue culture plates. At confluence, total RNA was prepared from the ES cells using a commercially available total RNA isolation system together with a Beckman FX robotic liquid handler (Beckman Coulter). The RNA samples were arrayed into a standard 96-well format and all subsequent manipulations were performed in 96-well plates.

The protocol by Townley et al. was used as a starting point for optimization, and modifications to the published protocol are shown in Figure 8.11.1. The primer sequences used are listed in Table 8.11.1.

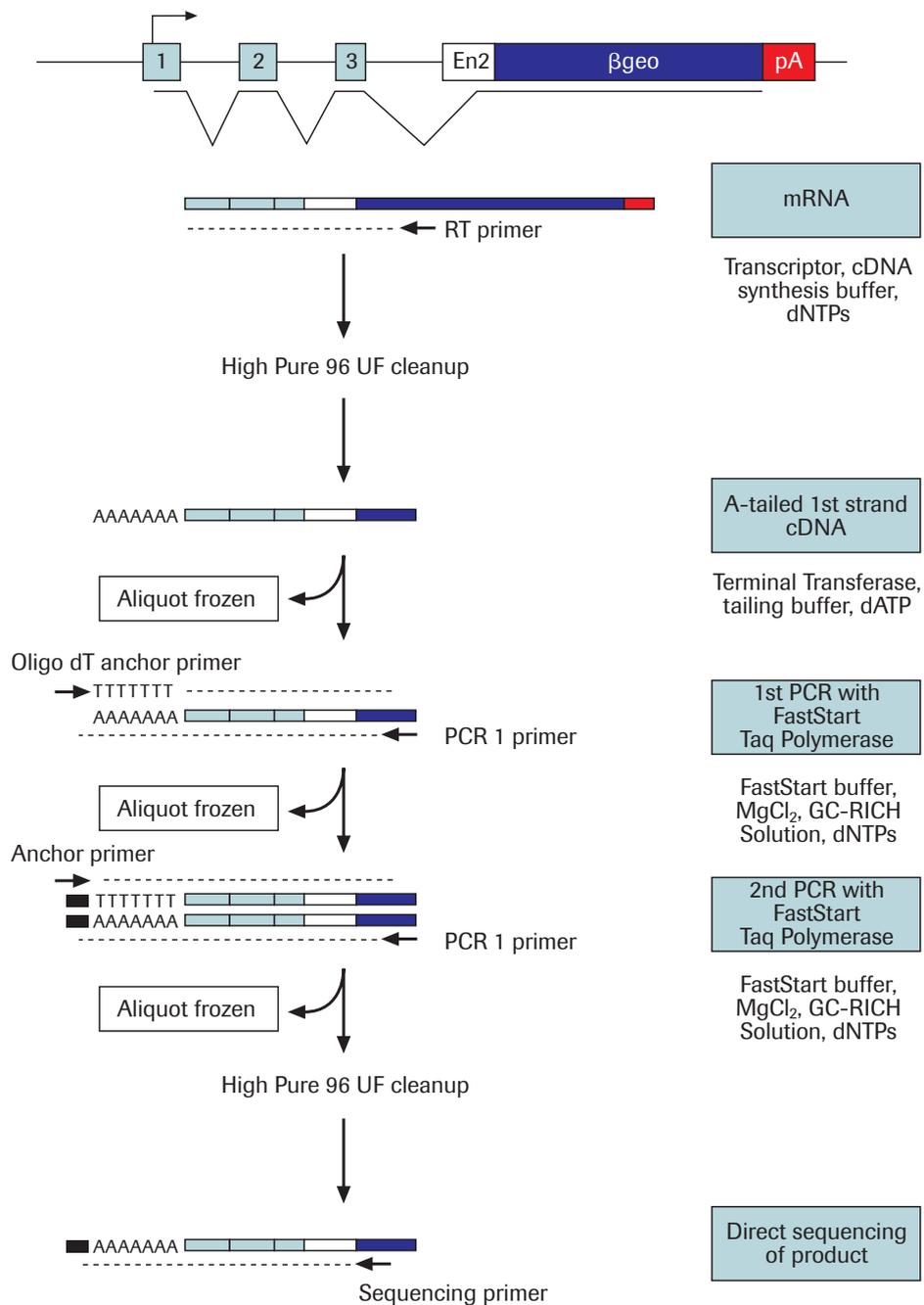


Fig. 8.11.1



<b>Table 1: Primer sequences used in the HTS 5' RACE kit</b>		
<b>Step</b>	<b>Primer name</b>	<b>Sequence (5' to 3')</b>
First strand cDNA synthesis	RT primer	TAATGGGATAGGTCACGT
First-round PCR	oligo dT anchor primer	GGTTGTGAGCTCTTCTAGATGGTTTTTTTTTTTTTTTTTT
First-round PCR	PCR 1 primere	AGTATCGGCCTCAGGAAGATCG
Second-round PCR	anchor primer	GGTTGTGAGCTCTTCTAGATGG
Second-round PCR	PCR 2 primere	PCRATTCAGGCTGCGCAACTGTTGGG
Sequencing	sequencing primer	CGACGGGATCCTCTAGAGT

**Table 8.11.1**

First strand cDNA synthesis was performed in a 20- $\mu$ l volume using 5  $\mu$ g (standard deviation of 2.45  $\mu$ g) of total RNA as the template, 12.5 pmol of RT primer, 1 mM dNTPs, and 25 units of Transcriptor Reverse Transcriptase in the recommended buffer at 55°C for 60 minutes, followed by a 5-minute incubation at 80°C.

Single-stranded cDNA was purified with HIGH PURE 96 UF Cleanup Plates by applying a vacuum (-400 to -600 mbar) through a Whatman Univac vacuum manifold for 10–15 minutes until dry, and then applying the vacuum for a further minute. The samples were resuspended in 30  $\mu$ l PCR-grade water on a microplate shaker for 5 minutes with moderate shaking (700 rpm). Then, 19  $\mu$ l of the samples were removed and placed into a new 96-well plate.

Prior to the cDNA tailing reaction, the samples were denatured at 94°C for 3 minutes, and then placed on ice. Eighty units of recombinant Terminal Transferase, 200 µM dATP and the recommended buffer were mixed in a volume of 6 µl and immediately added to the denatured cDNA, mixed, and incubated at 37°C for 20 minutes, followed by 10 minutes at 70°C. For the first round of PCR, 5 µl of the reaction was used and the remaining DNA was stored at -20°C.

Two rounds of nested PCR were performed, each using 1 unit of FastStart Taq DNA Polymerase in a total volume of 25 µl. For the first round, samples were denatured at 95°C for 5 minutes and then subjected to 40 cycles of denaturation (95°C for 30 seconds), annealing (55°C for 30 seconds) and extension (72°C for 2 minutes) using 18.75 pmol of oligo dT anchor primer, 6.25 pmol of PCR 1 primer and dNTPs to a final concentration of 200 µM in the appropriate buffer with MgCl<sub>2</sub> and GC-RICH solution.

For the second PCR step, 1 µl of the first round PCR product was taken and the remaining DNA was stored at -20°C.

For the second round, samples were amplified as described above at an annealing temperature of 60°C using 6.25 pmol of the anchor and PCR 2 primers.

PCR products were then dephosphorylated by adding 1 unit of recombinant alkaline phosphatase to the reaction and incubating at 37°C for 60 minutes, followed by 15 minutes at 70°C. These products were then purified with HIGH PURE 96 UF Cleanup Plates as described above.

Subsequently, 2 µl of this reaction was used as the template for a 10-µl cycle sequencing reaction (initial denaturation at 96°C for 30 seconds followed by 45 cycles of denaturation [92°C for 15 seconds], annealing [52°C for 15 seconds] and extension [60°C for 2 minutes]) in buffer containing 1% glycerol, 20 pmol of the sequencing primer, and 0.5 µl BigDye v3.1 (ABI). Following ethanol precipitation, RACE products were run on an ABI 3730 capillary sequencer and the sequences were analysed and annotated to the Ensembl gene build of the NCBI m33 assembly ([www.ensembl.org/Mus\\_musculus/](http://www.ensembl.org/Mus_musculus/)) using Wellcome Trust Sanger Institute software (ASP – Automated Sequence Preprocessing and MapTag, our custom annotation software).

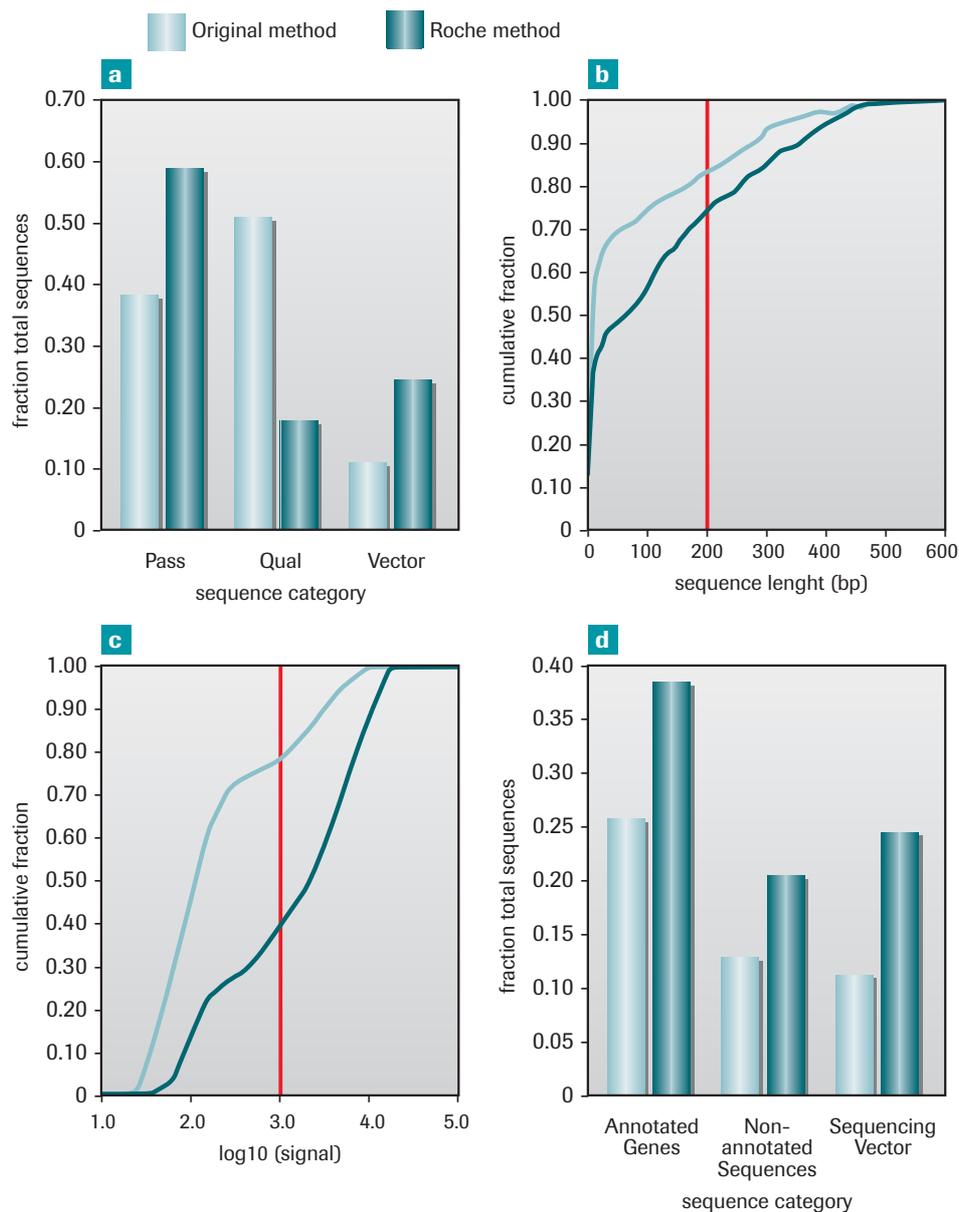
## Results and Applications

The protocol by Townley *et al.* is a robust method that has been used successfully to characterize many hundreds of gene trap cell lines. Approximately 60% of gene trap cell lines produce high quality sequence tags using this method (Townley *et al.* and unpublished results). However, multiple size selection steps carried out on individual microdialysis filters are not well-suited for high-throughput 5' RACE in a 96-well format. Our attempts to substitute microdialysis for other methods such as size exclusion columns have increased the throughput of the method, but at a lower efficiency: only 20–40% of gene trap cell lines produced high-quality sequence tags. Therefore, in close collaboration with Roche Diagnostics, we set out to develop a method that could be carried out in 96-well plates without a reduction in overall efficiency.

For the evaluation of the HTS 5' RACE kit<sup>1</sup>, 576 RNA samples were used and reactions were compared with reactions using our method based on the protocol by Townley *et al.*.

Four criteria – quality, length, signal intensity, and annotation – were used for our analysis of the sequence tags generated by 5' RACE and a marked improvement was observed for all criteria using the HTS 5' RACE kit<sup>1</sup> (Figure 8.11.2).

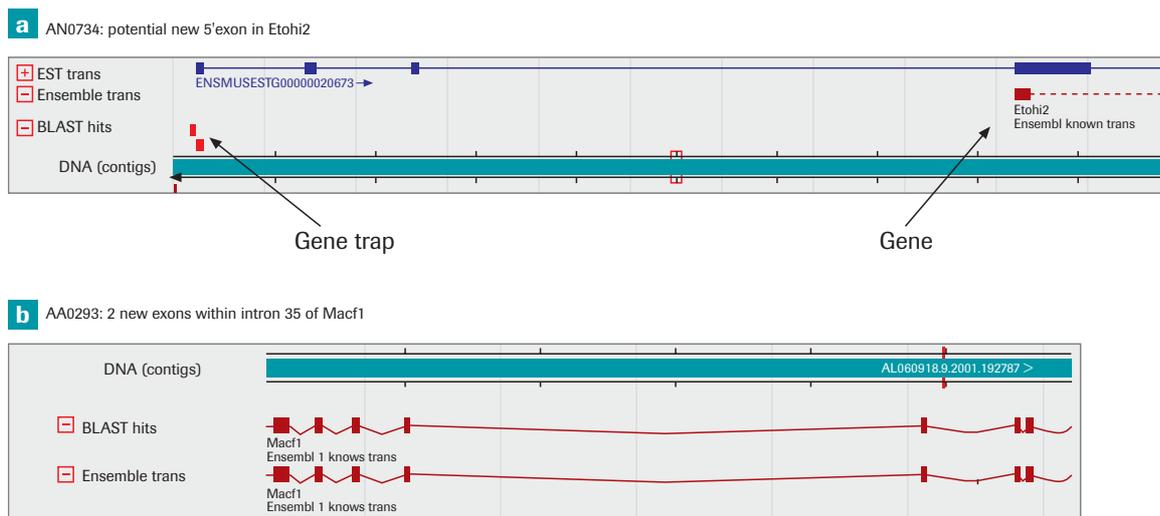


**Fig. 8.11.2**

The automated sequence analysis software (ASP) designates high-quality sequences as “Pass”, vector-derived sequences as “Vector”, and poor-quality sequences as “Qual”. Using the HTS 5' RACE kit<sup>1</sup>, 58% of the reactions produced high-quality sequence tags and fewer sequences failed (18%) compared with our original protocol (38% and 51%, respectively). Length analyses were performed on sequences following automated vector trimming where the base quality score using Phred was above 15. The average sequence length increased from 77 base pairs (bp) to 123 bp with the new method. Sequence lengths were also plotted as the cumulative fraction of the sequence length distribution (Figure 8.11.2b) and more of the resulting sequences are longer, for example, 27% of the sequences with the new protocol exceed 200 bp compared with 17% using the old method. On average, signal strengths (the average signal detected for a given nucleotide) were four times higher with the new method (3516 units) than with the original method (878 units). The signal intensity, plotted as a cumulative fraction against the log<sub>10</sub> of the signal (Figure 8.11.2c), shows that the new method outperforms the original method as 60% of the sequences have an intensity of more than 1000 units compared with only 22% using the original protocol.

The improvement in sequence quality, length and signal resulted in a significant improvement in the annotation of these tags. We obtained a 50% increase in annotation to known Ensembl genes, or RefSeq cDNAs (Figure 8.11.2d) and a 60% increase in the number of high quality sequences that match the unannotated sequences in the genome.

These “novel” sequences are likely to represent insertions in novel transcribed genes or to exons of known genes that are not yet annotated such as the 5' exon-most exons of genes or alternatively-spliced exons (Figure 8.11.3). Thus, the HTS 5' RACE kit\* will substantially facilitate gene annotation.



**Fig. 8.11.3**

### Summary

The Roche HTS 5' RACE kit\* has been developed for highthroughput 5' RACE in a 96-well format without a loss in overall efficiency. This kit was applied to the characterization of gene trap insertions in mouse ES cell lines and produced high quality sequence tags for 60% of gene trap cell lines, comparable to the previously published, lowerthroughput method. The HTS 5' RACE kit\* will greatly aid large-scale gene trapping efforts currently underway to generate insertional mutations in most genes in mouse ES cells. Equally, this method can be applied on a genome-wide scale to help characterize all transcribed exons that comprise the mammalian transcriptome.

### Products from Roche Applied Science used to generate these results:

FastStart Taq DNA Polymerase, Transcriptor Reverse Transcriptase, Terminal Transferase, recombinant, Deoxynucleoside Triphosphate Set, PCR Grade, Water, PCR Grade, HIGH PURE 96 UF Cleanup Plates, Geneticin, Alkaline Phosphatase, recombinant



For information on ordering these products, see the Appendix.

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For Pwo DNA Polymerase, Cat. Nos. 11 644 947 001, 22 644 955 001; Pwo SuperYield DNA Polymerase, Cat. Nos. 04 340 868 001, 04 340 850 001, see Disclaimer No. 2,

For the PCR Master, Cat. No. 11 636 103 001 see Disclaimer No. 3.

For the LightCycler<sup>®</sup> Probe Design Software 2.0, Cat. No. 04 342 054 001, see Disclaimer No. 8.



*Chapter 9*  
**Appendix**



9

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<b>9</b>	<b>Appendix</b>	<b>Page</b>
A	Troubleshooting.....	285
B	General Information.....	293
C	Ordering Information.....	299
D	Abbreviations.....	315
E	References.....	318
F	Index.....	325
G	Trademarks and License Disclaimers.....	334

## 9. Appendix

### A. Troubleshooting

#### Optimization of Conventional PCR (with Taq DNA Polymerase)

 You can also use these troubleshooting tips for reactions involving the Expand High Fidelity PCR System and the GC-RICH PCR System.

Observation	Possible Cause	Recommendation
Little or no PCR product	Pipetting errors, e.g.: <ul style="list-style-type: none"> <li>▶ Omission of a reagent</li> <li>▶ Enzyme added to master mix before buffer</li> </ul>	Repeat the PCR with the same sample and PCR reagents, but be sure to: <ul style="list-style-type: none"> <li>▶ Check all reagent concentrations.</li> <li>▶ Mix your reagent solutions before adding to master mix.</li> </ul>
	Multiple contributing factors	<ul style="list-style-type: none"> <li>▶ Repeat reaction with positive control template and established primers that have worked in previous experiments.</li> <li>▶ Start over, but use freshly made solutions of reaction buffer components, template, primers, and enzyme.</li> <li>▶ Use autoclaved tubes and contaminant-free pipets and tips</li> <li>▶ Ensure that the work bench is not contaminated</li> <li>▶ If using a thermal cycler with a heated lid, do not overlay the PCR samples with oil. The combination of a heated lid and an oil overlay may decrease the yield of PCR product.</li> <li>▶ Check gel analysis and staining procedure</li> </ul>
	Storage conditions	<ul style="list-style-type: none"> <li>▶ Check storage conditions of reagents and enzyme.               <ul style="list-style-type: none"> <li>! <i>Polymerase activity is very stable but may decrease after repeated freezing and thawing.</i></li> </ul> </li> </ul>
	Difficult template e.g., GC-rich templates	<ul style="list-style-type: none"> <li>▶ Add DMSO (at various concentrations up to 10%) to the reaction mix and use less enzyme (starting with as little as 0.5 U per reaction).               <ul style="list-style-type: none"> <li>! <i>DMSO may reduce the accuracy of the reaction.</i></li> </ul> </li> <li>▶ Use the GC-RICH PCR System and include GC-RICH resolution solution (varying from 0.5 - 2.5 M, in steps of 0.25 M) in the reaction.</li> <li>▶ Make sure you use the optimal enzyme for your application</li> </ul>
	DNA template quality poor (degraded, contaminated, contains inhibitors)	<ul style="list-style-type: none"> <li>▶ Check quality and concentration of template:</li> <li>▶ Analyze an aliquot on an agarose gel to check concentration. If concentration is too low, use more template.</li> <li>▶ Amplify template with an established primer pair or in an established PCR system.</li> <li>▶ Check purity of template on a gel. If template is impure or degraded, repeat purification. For example, prepare new template with the High Pure PCR Template Preparation Kit, which produces high molecular weight, ultrapure DNA.</li> <li>▶ If template was degraded, store new template at 4°C.               <ul style="list-style-type: none"> <li>! <i>For long term storage, freeze the template and store at -20°C. However, avoid repeated freezing and thawing.</i></li> </ul> </li> <li>▶ Try various Mg<sup>2+</sup> concentrations (between 1.5 and 5 mM).</li> <li>▶ Try additives such as DMSO and detergents to increase efficiency of reaction.</li> <li>▶ Crude DNA extracts may degrade over time, and contain PCR inhibitors; try diluting DNA samples (unless target is a single copy region). For a subset of the crude extracts, try e.g., a series of dilutions (10-, 50-, 200-, 500-fold).</li> <li>▶ RNA (e.g., 18S gene) may inhibit PCR. Pre-treat sample with RNase.</li> <li>▶ Use primers that amplify smaller targets.</li> </ul>
	Enzyme concentration or activity too low	<ul style="list-style-type: none"> <li>▶ Increase enzyme concentration (in 0.5 U steps).</li> <li>▶ Check polymerase activity with positive control template and primers.</li> <li>▶ Use a new batch of polymerase.</li> <li>▶ Increase number of cycles.</li> <li>▶ Use an enzyme blend, such as Expand High Fidelity PCR System.</li> </ul>



Observation	Possible Cause	Recommendation
<b>Little or no PCR product</b>	Reaction buffer not optimized or dNTP/MgCl <sub>2</sub> concentration too low	<ul style="list-style-type: none"> <li>▶ Both EDTA and dNTP will chelate Mg<sup>2+</sup> and lower its effective concentration in the reaction. Check EDTA levels in the DNA sample; they should never be greater than 0.1 mM.</li> <li>▶ Many 10× commercial buffers contain 10 mM Mg<sup>2+</sup>; this is often too low for optimal amplification. Increase the MgCl<sub>2</sub> concentration in steps of 0.25 mM. (1.5 mM MgCl<sub>2</sub> is the minimum concentration, use up to 3.5 mM.)</li> <li>▶ Run a side-by-side comparison of PCRs with new tube of 10× dNTPs and your old tube of dNTPs.</li> <li>▶ If you increase the dNTP concentration, also increase the Mg<sup>2+</sup> concentration.</li> <li>▶ Use the PCR Optimization Kit to optimize reaction.</li> </ul>
	Cycle conditions not optimal	<p>Check cycle conditions:</p> <ul style="list-style-type: none"> <li>▶ In General, keep initial denaturation short</li> <li>▶ Increase initial denaturation step if necessary (<i>e.g.</i>, use 3 min at 95°C).</li> <li>▶ Check denaturation temperature. (It should be between 90°C and 97°C.)</li> <li>▶ Decrease annealing temperature.</li> <li>▶  <i>To determine the optimal annealing temperature, perform touchdown PCR.</i></li> <li>▶ Increase the extension time (in increments of 1 minute).</li> <li>▶ Increase number of cycles (up to 40) and/or amount of template DNA.</li> <li>▶ Make sure that the final elongation step is included in the program (5 min recommended).</li> <li>▶ Check performance of different regions of the thermal block.</li> </ul>
Primer design not optimal	<ul style="list-style-type: none"> <li>▶ Verify primer sequence. For example a mismatch at the 3' end of the primer will lead to reduced or no product.</li> <li>▶ Design alternative primers.</li> <li>▶ Use primer design programs</li> <li>▶ Avoid GC-rich stretches</li> <li>▶ Make sure that forward and reverse primer have similar <math>T_m</math>.</li> </ul>	
Primer concentration not optimal	<ul style="list-style-type: none"> <li>▶ Check the concentration of your primers by measuring <math>A_{260\text{ nm}}</math>. Both primers should be present in the reaction at the same concentration.</li> <li>▶ Try various primer concentrations (between 0.1 and 0.6 μM each).</li> <li>▶ If this reaction involved a new batch of primers or a new dilution of primers, try an older batch or dilution.</li> </ul>	
Annealing temperature too high	<ul style="list-style-type: none"> <li>▶ Reduce annealing temperature; initially, try lowering it 5°C. (Minimal annealing temperature normally is 55°C but you may try 40–45°C.)</li> <li>▶ Determine the optimal annealing temperature by touch-down PCR.</li> </ul>	
Primer specificity not optimal	Perform nested PCR with nested primers.	
Primer quality or storage problems	<ul style="list-style-type: none"> <li>▶ If you use an established primer pair, check their performance in an established PCR system (<i>i.e.</i>, with a control template).</li> <li>▶ Make sure that the primers are not degraded.</li> <li>▶ Always store primers at –15 to –25°C.</li> </ul>	
Formation of primer-dimers	<ul style="list-style-type: none"> <li>▶ Use two separate master mixes for reaction set-up:</li> <li>▶ Mix 1: dNTPs, template DNA and water up to 25 μl</li> <li>▶ Mix 2: all other components and water up to 25 μl</li> <li>▶ Combine Mixes 1 and 2, mix thoroughly, centrifuge briefly and transfer the reactions immediately into PCR instrument.</li> <li>▶ Avoid self-complementarity (primer dimer formation)</li> <li>▶ Use FastStart (instead of regular) Taq DNA Polymerase.</li> </ul>	



Observation	Possible Cause	Recommendation
<b>Multiple bands or background smear</b>	Annealing temperature too low	▶ Increase annealing temperature (depending on primer length, maximum is 68°C).
	Nonspecific binding of primers	▶ Check and optimize primer concentration. ▶ Use "Hot Start" reaction.
	Primer design or concentration not optimal	▶ Redesign primers for more specific binding of target and/or to allow a higher annealing temperature. ▶ Try various primer concentrations (between 0.1 and 0.6 μM each). ▶ Both primers should be present in the reaction at the same concentration. ▶ Perform nested PCR with nested primers.
	Difficult template e.g., GC-rich template	Addition of meltin temperature reducing components e.g., ▶ Add GC-RICH solution. ▶ Add DMSO (2-10%). ▶ Add glycerol (5-25%). ▶ Add betain (0.5-2 M). ▶ Add urea
	Too much starting template	Make serial dilutions of template and perform PCR with each dilution.
<b>PCR products in negative control experiments</b>	Secondary amplification product	▶ Check reagent concentrations and cycle conditions. ▶ Optimize Mg <sup>2+</sup> concentration. ▶ Verify/optimize primer concentration. ▶ Decrease number of cycles. ▶ Verify (and perhaps decrease) concentration of template. ▶ Use less enzyme in reaction.
	Carryover contamination	▶ Replace all reagents, especially water. ▶ Use disposable aerosol-resistant pipette tips that contain hydrophobic filters. ▶ Use separate work areas for DNA extraction, pre-PCR set up and post-PCR examination. ▶ Do not move equipment like pipettes, racks, microfuges between separate work areas. ▶ Avoid creating aerosols (e.g., when ejecting pipette tips). ▶ Use UV lamps to decontaminate the laboratory and the laminar flow hood whenever possible. ▶ Include dUTP (600 μM) instead of dTTP (200 μM) in reaction, then pre-treat subsequent PCRs with thermolabile UNG (1 U/50 μl reaction). <b>!</b> <i>Increase Mg<sup>2+</sup> concentration to a maximum of 4 mM (in steps of 0.25 mM) to compensate for higher dNTP concentration. See section 4.6 in this manual for more information.</i>



### Optimization of Hot Start PCR (with FastStart Taq DNA Polymerase)



You can also use these troubleshooting tips for reactions involving the FastStart High Fidelity PCR System.

Observation	Possible Cause	Recommendation
<b>Little or no PCR product</b>	FastStart Taq DNA Polymerase not fully activated	<ul style="list-style-type: none"> <li>▶ Make sure that the enzyme was activated at 95°C for 4 min prior to PCR cycles.</li> <li>▶ For difficult templates, increase activation time to 10 minutes.</li> <li>▶ Check denaturation time during cycles. It should be a minimum of 30 seconds.</li> <li>▶ Check number of cycles in program. Increase the number of cycles (in steps of 5 cycles).</li> </ul>
	Pipetting errors	Check all concentrations and storage conditions of reagents, then repeat PCR.
	Mg <sup>2+</sup> concentration not optimal	Adjust Mg <sup>2+</sup> concentration in 0.5 mM steps. (Try various concentrations between 1 and 4 mM.)
	Primer problems due to: <ul style="list-style-type: none"> <li>▶ suboptimal design</li> <li>▶ concentration</li> <li>▶ quality or storage problems</li> <li>▶ annealing temperature too high</li> </ul>	<ul style="list-style-type: none"> <li>▶ If you are using an established primer pair, check their performance on a well-defined control template (in an established PCR system).</li> <li>▶ Try various primer concentrations (between 0.2 and 0.5 μM each).</li> <li>▶ Reduce annealing temperature.</li> <li>▶ Design alternative primers.</li> <li>▶ Try nested PCR with nested primer pairs.</li> </ul>
	DNA template problems	Check quality/ concentration of template: <ul style="list-style-type: none"> <li>▶ Analyze an aliquot on a agarose gel. If impure or degraded, repurify template.</li> <li>▶ Use serial dilutions of template in PCR.</li> <li>▶ Amplify template with an established primer pair or in an established PCR system.</li> </ul>
	Cycle conditions are not optimal	<ul style="list-style-type: none"> <li>▶ Decrease annealing temperature.</li> <li>▶ Check elongation time (should be 1 min/1 kb target length).</li> <li>▶ Denaturation time should not be less than 30 seconds at 95°C.</li> <li>▶ Increase number of cycles.</li> </ul>
	Difficult template, <i>e.g.</i> , GC-rich	<ul style="list-style-type: none"> <li>▶ Repeat PCR under same conditions, but add 5 μl GC-RICH solution (included with FastStart Taq DNA Polymerasetaq dna polymerase) to reaction.</li> <li>▶ If result is still not satisfactory, try using different amounts of GC-RICH solution (<i>e.g.</i> 4, 6 or 8 μl).</li> <li>▶ Try optimizing other parameters (<i>e.g.</i>, Mg<sup>2+</sup> concentration, enzyme amount, annealing temperature).</li> </ul>
<b>Multiple bands or background smear</b>	Too much: <ul style="list-style-type: none"> <li>▶ Mg<sup>2+</sup></li> <li>▶ Template</li> <li>▶ Enzyme</li> </ul>	<ul style="list-style-type: none"> <li>▶ Reduce Mg<sup>2+</sup> concentration.</li> <li>▶ Check template concentration on an agarose gel. Try various lower template concentrations.</li> <li>▶ Reduce enzyme amount in 0.25 U steps.</li> </ul>
<b>Other problems</b>	Causes common to all PCRs	See "Optimization of Standard PCR" above for recommendations.
<b>Problems with cloning of PCR products</b>		<ul style="list-style-type: none"> <li>▶ FastStart Taq DNA Polymerase adds an additional A at the 3' end of PCR products (just like Taq DNA Polymerase). Therefore, try cloning PCR products into TA cloning vectors.</li> <li>▶ Before cloning into blunt-ended vectors, "polish" the ends of the PCR products.</li> </ul>



## Optimization of Long Template PCR

Observation	Possible Cause	Recommendation
<b>Little or no PCR product</b>	Difficult template	<ul style="list-style-type: none"> <li>▶ If using the Expand Long Template PCR System or Expand LongRange dNTPack, try using amplification system 2 or 3, even if you have short amplicons.</li> <li>▶ Use only highly purified template preparations.</li> <li>▶ If possible, linearize circular templates.</li> </ul>
	Mg <sup>2+</sup> concentration too low	<p>Increase the MgCl<sub>2</sub> concentration in 0.25 mM steps.</p> <p> For Expand Long Range dNTPack, the minimum MgCl<sub>2</sub> concentration is 1.75 mM; for Expand 20 kb<sup>PLUS</sup> PCR System, it is 2.75 mM.</p>
<b>Other problems</b>	Causes common to all PCRs	See "Optimization of Standard PCR" above for recommendations.

### Optimization of Two-Step RT-PCR

Observation	Possible Cause	Recommendation
<b>Little or no product in cDNA reaction</b>	Too little RNA template	<ul style="list-style-type: none"> <li>▶ Check quality and concentration of template:</li> <li>▶ Analyze an aliquot on a denaturing agarose gel to check for possible degradation or impurity. If degraded or impure, repeat purification.</li> <li>▶ Amplify template with an established primer pair or in an established RT-PCR system.</li> <li>▶ Determine concentration of RNA template by measuring <math>A_{260}</math>.</li> <li>▶ Use purified mRNA rather than total RNA as template.</li> <li>▶ Use 10 ng - 5 µg of total RNA (or 1 - 100 ng of mRNA). If you must use lower amounts of RNA, try priming with a gene-specific primer.</li> <li>! <i>Add 10 µg/ml MS2 RNA to template to stabilize low concentrations of target RNA.</i></li> </ul>
	Too much template RNA	<ul style="list-style-type: none"> <li>▶ Decrease amount of RNA template. Too much RNA may affect/inhibit performance of RT reaction.</li> </ul>
	RNase contamination	<ul style="list-style-type: none"> <li>▶ Protect RNA from ribonuclease degradation by adding Protector RNase Inhibitor to the cDNA reaction.</li> <li>! <i>Inhibitor concentrations up to 60 U will not interfere with RT-PCR.</i></li> <li>▶ Use RNase-free tubes and pipet tips.</li> </ul>
	Difficult template with secondary structure (GC-rich templates)	<ul style="list-style-type: none"> <li>▶ RNA templates up to 70% GC content can be reverse transcribed if you:</li> <li>▶ Denature the template-primer mixture for 10 min at 65°C before adding reverse transcriptase.</li> <li>▶ Use random hexamer primers or a gene-specific primer.</li> <li>▶ Use Transcriptor Reverse Transcriptase to reverse transcribe at temperatures as high as 65°C.</li> </ul>
	Enzyme concentration too high or low	<ul style="list-style-type: none"> <li>▶ Do not use more than 10 U Transcriptor Reverse Transcriptase to transcribe 1 µg total RNA template in a 20 µl cDNA synthesis reaction.</li> <li>▶ For &gt;1 µg total RNA, increase reaction volume and amount of Transcriptor Reverse Transcriptase proportionally.</li> <li>▶ For low template concentrations, use less reverse transcriptase.</li> </ul>
	Reaction temperature too high or low	<ul style="list-style-type: none"> <li>▶ Perform the RT reaction (for templates up to 4 kb) for 30 min at a temperature between 42°C and 65°C.</li> <li>▶ For transcripts &gt;4 kb, perform the reaction at a temperature between 50°C and 60°C for 1 h.</li> <li>! <i>Prolonged incubation at lower temperatures will increase the yield of full-length product.</i></li> </ul>
	Wrong gene-specific primer	<ul style="list-style-type: none"> <li>▶ Try another gene-specific primer or switch to an anchored oligo(dT) primer [e.g., the anchored oligo(dT)<sub>18</sub> primer included in the Transcriptor First Strand cDNA Synthesis Kit].</li> <li>▶ Make sure that the gene-specific primer is able to bind to the mRNA (antisense direction).</li> </ul>
	Inhibitors of RT reaction	<p>Remove inhibitors by precipitating the mRNA, washing the precipitate with 70% ethanol, then redissolving the precipitate.</p> <p>! <i>Remove all traces of ethanol before using RNA in RT.</i></p>



Observation	Possible Cause	Recommendation
<b>Little or no PCR product</b>	Contamination by genomic DNA	<ul style="list-style-type: none"> <li>▶ Design primers that recognize different exons, so they can distinguish between genomic DNA contaminants and cDNA.</li> <li>▶ Always include a “no RT” control that did not contain reverse transcriptase during the cDNA synthesis step.</li> </ul>
	MgCl <sub>2</sub> concentration for PCR too low or high	<ul style="list-style-type: none"> <li>▶ Optimize the MgCl<sub>2</sub> concentration of the PCR reaction.</li> <li>! <i>Each μl of the 20 μl cDNA reaction contributes 0.4 mM MgCl<sub>2</sub> to the subsequent PCR reaction.</i></li> <li>▶ Optimize MgCl<sub>2</sub> concentration for each template and primer combination.</li> </ul>
	Annealing temperature too low	Increase annealing temperature to accommodate the melting temperature of the primers.
	Primer design for PCR not optimal	<ul style="list-style-type: none"> <li>▶ Design alternative primers.</li> <li>▶ Both primers should have similar melting temperatures.</li> </ul>
	Primer concentration in PCR not optimal	<ul style="list-style-type: none"> <li>▶ Both primers should be present in the reaction at the same concentration.</li> <li>▶ Try various primer concentrations (between 0.1 and 0.6 μM for each primer).</li> <li>▶ Check for possible degradation of the primers (e.g., on a denaturing polyacrylamide gel or by HPLC).</li> </ul>
	Too much cDNA inhibits PCR	The volume of cDNA template (from the RT reaction) should not exceed 10% of the total volume of the PCR reaction.
Formation of primer-dimers	<ul style="list-style-type: none"> <li>▶ Use FastStart Taq DNA Polymerase or FastStart High Fidelity PCR System.</li> <li>▶ Design primers that do not contain complementary sequences.</li> <li>▶ Make sure a denaturation step is included at the end of the cDNA synthesis reaction (5 min at 85°C).</li> </ul>	

### Optimization of One-Step RT-PCR

Observation	Possible Cause	Recommendation
<b>Little or no PCR product</b>	Difficult RNA template (e.g., with GC content >60%)	<ul style="list-style-type: none"> <li>▶ Briefly denature the RNA template at 94°C (1 min) before adding it to reaction mixture.</li> <li>! <i>Do not incubate RTase or RNase inhibitor at this elevated temperature (even briefly), since they will be inactivated.</i></li> <li>▶ Increase denaturation temperature or time in PCR cycles.</li> <li>▶ If using the C. therm Polymerase One-Step RT-PCR System, optimize the concentration of DMSO (up to 10% maximum).</li> </ul>
	Other RNA template problems	See “Optimization of Two-Step RT-PCR” above for recommendations.
<b>Other PCR problems</b>	Causes common to all PCRs	See “Optimization of Standard PCR” above for recommendations.



### Optimization of Multiplex PCR

Observation	Possible Cause	Recommendation
<b>PCR doesn't work with a cDNA template</b>	Hexamer primers in the RT reaction can interfere with subsequent PCR	Use anchored oligo(dT) primer in RT reaction rather than hexamer primers.
	RNA is degraded	Check quality of RNA on a gel. Repurify if degraded.
<b>Nonspecific product bands</b>	Nonspecific binding of primers	Redesign primers to get more specific binding to target and/or to allow a higher annealing temperature.
	Formation of primer-dimers	<ul style="list-style-type: none"> <li>▶ Increase annealing temperature.</li> <li>▶ Check primer set for cross-complementarities. If necessary, redesign primers (e.g., with LightCycler® Primer Design Software 2.0.</li> </ul>
	Priming by shorter fragments that are impurities in the primer preparation	Use only HPLC-purified primers.
<b>Difficult amplicons are used and not all PCR products are amplified</b>	Activation time of FastStart High Fidelity enzyme blend is too short	Increase activation time to 10 min.
	Primer design not optimal	Redesign primers. (See above.)
	Primer concentration not optimal	Try a different concentration of primers.
	Too little enzyme blend in reaction	Increase the amount of enzyme and MgCl <sub>2</sub> (e.g., 3.5 U FastStart High Fidelity enzyme blend and 3.5 mM MgCl <sub>2</sub> ) to get higher specificity and yield.
<b>Product bands cannot be separated on gel</b>	Sizes of amplicons are too close together	Redesign primer sets.
	Incorrect concentration of agarose in gel	<ul style="list-style-type: none"> <li>▶ Use a 3-4% agarose gel to separate products that are as little as 20-50 bp apart.</li> <li>▶ Include a positive control mix (3 µl of each monoplex product) on the gel.</li> </ul>
<b>Faint bands visible</b>	Too little PCR product was loaded on gel	Load more product on each gel lane.
<b>Multiplex PCR does not give a satisfactory result in the presence of additives</b>	Too little FastStart High Fidelity enzyme blend added to reaction	Increase the amount of enzyme and MgCl <sub>2</sub> (e.g., 3.5 U FastStart High Fidelity enzyme blend and 3.5 mM MgCl <sub>2</sub> ) to get higher specificity and yield.



## B. General Information

- ▶ Metric Prefixes (International System)
- ▶ Moles , Molar, Molarity
- ▶ Nucleic Acids
  - ▶ Conversions and Calculations
  - ▶ Content and Distribution

### Metric Prefixes (International System)

Symbol	Prefix	Multiplication factor	Symbol	Prefix	Multiplication factor
T	tera	$10^{12}$	m	milli	$10^{-3}$
G	giga	$10^9$	$\mu$	micro	$10^{-6}$
M	mega	$10^6$	n	nano	$10^{-9}$
k	kilo	$10^3$	p	pico	$10^{-12}$
			f	femto	$10^{-15}$
			a	atto	$10^{-18}$

### Moles , Molar, Molarity

Term	Symbol	Meaning	Examples
Mole	mol	Absolute amount of a substance	1 mol = $6.022 \times 10^{23}$ molecules
Molar or Molarity	mol/l or M	Concentration of a substance in a liquid Molarity = $\frac{\text{moles of substance}}{\text{liter of solution}}$	a) 1 mol/l = $6.022 \times 10^{23}$ molecules/liter = a one molar solution = a solution with a molarity of one b) A solution with 0.20 pmol in 100 $\mu$ l has a concentration of 2 nM. [Calculation: 0.20 pmol in 100 $\mu$ l = 2 pmol in 1 ml = 2 nmol in 1 l (2 nM)]
Molar weight	g/mol	Weight of 1 mol (= $6.023 \times 10^{23}$ parts) of a molecule, as defined by the molecular weight of this molecule.	Molecular weight of Tris: 121.1 g/mol. An 1 M (mol/liter) solution of Tris H <sub>2</sub> O is prepared by dissolving 121.1 g Tris in H <sub>2</sub> O to a final volume of 1 liter.



## Nucleic Acids

### Conversions and Calculations

#### Spectral constants for nucleotides<sup>1</sup>

Compound	Molecular weight (MW)	~ max (pH 7.0)	Absorbance at ~ max (1 M solution)
ATP	507.2	259	15,400
dATP	491.2	259	15,400
CTP	483.2	271	9,000
dCTP	467.2	272	9,100
GTP	523.2	253	13,700
dGTP	507.2	253	13,700
UTP	484.2	260	10,000
dTTP	482.2	267	9,600



Molar concentration of nucleic acid = (observed absorbance at  $\lambda_{max}$ )  $\div$  absorbance at  $\lambda_{max}$  for 1 M solution.

#### Spectrophotometric equivalents<sup>2</sup>

1 $A_{260}$ unit	Nucleic acid	Amount	Molarity (in nucleotides)
	double-stranded DNA	50 $\mu$ g/ml	0.15 mM
	single-stranded DNA	33 $\mu$ g/ml	0.10 mM
	single-stranded RNA	40 $\mu$ g/ml	0.11 mM
	oligonucleotide <sup>a</sup>	20 – 30 $\mu$ g/ml	0.06 – 0.09 mM

<sup>a)</sup> For exact determination of the molecular weight, see table "Conversions between weight and molarity of various DNAs".

#### Determining purity of nucleic acid preparations

For pure	$A_{260}/A_{280}$ <sup>a</sup>
DNA	= 1.8
RNA	= 2.0

<sup>a)</sup> An  $A_{260}/A_{280}$  ratio of <1.8 (DNA) or <2.0 (RNA) means the nucleic acid preparation contains contaminants (e.g. protein or phenol).

### Calculating molecular weight of nucleic acids<sup>3</sup>

For molecular weight of	Use this calculation
DNA base pair (sodium salt)	1 base pair = 660 daltons
double-stranded DNA (dsDNA)	(number of base pairs) × (660 daltons/base pair)
single-stranded DNA (ssDNA)	(number of bases) × (330 daltons/base)
single-stranded RNA	(number of bases) × (340 daltons/base)
oligonucleotide	<b>For dephosphorylated oligonucleotides:</b> [(number of A × 312.2) + (number of G × 328.2) + (number of C × 288.2) + (number of T × 303.2)] - 61 <b>For phosphorylated oligonucleotides:</b> [(number of A × 312.2) + (number of G × 328.2) + (number of C × 288.2) + (number of T × 303.2)] + 17

### Calculating moles of ends

To calculate	For this DNA	Use this formula
Moles of ends	dsDNA	$[2 \times (\text{grams of DNA})] \div (\text{MW in daltons})$
Picomoles of ends per microgram	dsDNA	$(2 \times 10^6) \div (660 \times \text{number of bases})$
Moles of ends generated by restriction endonuclease cleavage	circular dsDNA	$2 \times (\text{moles of DNA}) \times (\text{number of sites})$
	linear dsDNA	$[2 \times (\text{moles of DNA}) \times (\text{number of sites})] + [2 \times (\text{moles of DNA})]$

### Converting between weight and molarity for dsDNA

To convert	Calculate*
pmol to $\mu\text{g}$	$\text{pmol} \times N \times 660 \text{ pg}/1 \text{ pmol} \times 1 \mu\text{g}/10^6 \text{ pg} = \mu\text{g}$
$\mu\text{g}$ to pmol	$\mu\text{g} \times 10^6 \text{ pg}/1 \mu\text{g} \times 1 \text{ pmol}/660 \text{ pg} \times 1/N = \text{pmol}$

\* N = number of base pairs in DNA; 660, average molecular weight of a base pair.

### Converting between weight and molarity for ssDNA

To convert	Calculate*
pmol to $\mu\text{g}$	$\text{pmol} \times N \times 330 \text{ pg}/1 \text{ pmol} \times 1 \mu\text{g}/10^6 \text{ pg} = \mu\text{g}$
$\mu\text{g}$ to pmol	$\mu\text{g} \times 10^6 \text{ pg}/1 \mu\text{g} \times 1 \text{ pmol}/330 \text{ pg} \times 1/N = \text{pmol}$

\* N = number of nucleotides in DNA; 330, average molecular weight of a nucleotide.



Conversions between weight and molarity for various DNAs<sup>3</sup>

Type	Size (bp)	Molecular weight <sup>a</sup> (daltons)	pmol/~g	Molecules/~g	~g/pmol
oligonucleotide (ssDNA)	20 (bases)	6600	152	$9.1 \times 10^{13}$	$6.6 \times 10^{-3}$
dsDNA <sup>b</sup>	1000	$6.6 \times 10^5$	1.52	$9.1 \times 10^{11}$	0.66
pUC18/19 DNA	2686	$1.8 \times 10^6$	0.56	$3.4 \times 10^{11}$	1.77
pBR322 DNA	4361	$2.9 \times 10^6$	0.35	$2.1 \times 10^{11}$	2.88
M13mp18/19 DNA	7250	$4.8 \times 10^6$	0.21	$1.3 \times 10^{11}$	4.78
~ DNA	48502	$3.2 \times 10^7$	$3.1 \times 10^{-2}$	$1.8 \times 10^{10}$	32.01
<i>Escherichia coli</i> genome	$4.7 \times 10^6$	$3.1 \times 10^9$	$3.2 \times 10^{-4}$	$1.9 \times 10^8$	$3.1 \times 10^3$
<i>Saccharomyces cerevisiae</i> haploid genome	$1.5 \times 10^7$	$9.9 \times 10^9$	$1.0 \times 10^{-4}$	$6.0 \times 10^7$	$9.9 \times 10^3$
<i>Mus musculus</i> (mouse) haploid genome	$2.7 \times 10^9$	$1.8 \times 10^{12}$	$5.6 \times 10^{-7}$	$3.4 \times 10^5$	$1.8 \times 10^6$
<i>Homo sapiens</i> (human) haploid genome	$3.3 \times 10^9$	$2.2 \times 10^{12}$	$4.6 \times 10^{-7}$	$2.8 \times 10^5$	$2.2 \times 10^6$
<i>Zea mays</i> (maize) haploid genome	$3.9 \times 10^9$	$2.6 \times 10^{12}$	$3.9 \times 10^{-7}$	$2.3 \times 10^5$	$2.6 \times 10^6$

<sup>a</sup>) Average weight of a base pair = 660.

<sup>b</sup>) Sample calculation for conversions between weight and molarity of DNA for a DNA of 1000 bp:

Molecular weight:  $1000 \text{ bp} \times 660 \text{ daltons/bp} = 660,000 \text{ daltons} = 6.6 \times 10^5 \text{ daltons}$

1 pmol ( $10^{-12} \text{ mol}$ ) =  $6.022 \times 10^{11} \text{ molecules} = 6.6 \times 10^5 \text{ pg}$

pmol/ $\mu\text{g}$ :  $10^6 \text{ pg}/\mu\text{g} \times 1 \text{ pmol}/(660 \times 1000) \text{ pg} = 1.52 \text{ pmol}/\mu\text{g}$

Molecules/ $\mu\text{g}$ :  $6.022 \times 10^{11} \text{ molecules}/\text{pmol} \times 1.52 \text{ pmol}/\mu\text{g} = 9.1 \times 10^{11} \text{ molecules}/\mu\text{g}$

$\mu\text{g}/\text{pmol}$ :  $(660 \times 1000) \text{ pg}/\text{pmol} \times 1 \mu\text{g}/10^6 \text{ pg} = 0.66 \mu\text{g}/\text{pmol}$

Formulas to calculate melting temperature  $T_m$ 

System <sup>a</sup>	Formula <sup>b</sup>
<b>DNA - DNA hybrids</b>	▶ $T_m = 81.5^\circ\text{C} + 16.6 \log[\text{Na}^+] + 0.41 (\%GC) - 0.61 (\%for) - 500/N$
<b>DNA - RNA hybrids</b>	▶ $T_m = 79.8^\circ\text{C} + 18.5 \log[\text{Na}^+] + 0.58 (\%GC) + 11.8 (\%GC)^2 - 0.50 (\%for) - 820/N$
<b>RNA - RNA hybrids</b>	▶ $T_m = 79.8^\circ\text{C} + 18.5 \log[\text{Na}^+] + 0.58 (\%GC) + 11.8 (\%GC)^2 - 0.35 (\%for) - 820/N$
<b>Oligonucleotides</b>	▶ For oligonucleotides 14 - 25 nucleotides in length: ▶ $T_m = [2^\circ\text{C} \times (\text{number of A and T bases})] + [4^\circ\text{C} \times (\text{number of G and C bases})]$ ▶ For Oligonucleotides longer than 25 nucleotides: see formula for DNA/DNA hybrids

<sup>a</sup> The proposed formula is valid for  $\text{Na}^+$  concentration between 0.01 - 0.4 M and %GC values between 30 - 75%

<sup>b</sup>  $[\text{Na}^+]$  = concentration of  $\text{Na}^+$  ions, %GC = percentage of G and C nucleotides in the nucleic acid, %for = percentage of formamide in the hybridisation solution, N = length of the duplex in base pairs

**% GC Content of different Genomes**

<b>Organism</b>	<b>%GC content</b>
<b>Phages</b>	
T2	34.6
T3	49.6
T7	47.4
Lambda	48.8
<b>Prokaryotes</b>	
Agrobacterium tumefaciens	58 - 59.7
Bacillus subtilis	42.6
Escherichia coli	51
Mycobacterium tuberculosis	65
Staphylococcus aureus	32.4 - 37.7
<b>Vertebrates</b>	
Homo sapiens	40.3
Xenopus laevis	40.9
Mus musculus	40.3
Rattus species	41.8

## Content and Distribution

### Nucleic acids in an average human cell

DNA	~6 pg/cell <sup>a</sup>
Coding sequences	3% of genomic DNA
Number of genes	0.5–1.0 × 10 <sup>5</sup>
Active genes	1.5 × 10 <sup>4</sup>
Total RNA	~10 – 50 pg/cell <sup>b</sup>
rRNAs	80 – 85% of total RNA
tRNAs, snRNAs, and low mol. wt. RNA	15 – 20% of total RNA
mRNAs	1 – 5% of total RNA
nuclear RNA	~14% of total RNA
Ratio of DNA:RNA in nucleus	~ 2:1
Number of mRNA molecules <sup>c</sup>	0.2 – 1.0 × 10 <sup>6</sup>
Number of different mRNA species	1.0 – 3.4 × 10 <sup>4</sup>
Low abundance mRNA (5 – 15 copies/cell)	11,000 different messages
Intermediate abundance mRNA (200 – 400 copies/cell)	500 different messages
High abundance mRNA (12,000 copies/cell)	<10 different messages
Abundance of each message for:	<0.004% of total mRNA
Low abundance mRNA (5 – 15 copies/cell)	<0.1% of total mRNA
Intermediate abundance mRNA (200 – 400 copies/cell)	3% of total mRNA
High abundance mRNA (12,000 copies/cell)	

<sup>a</sup> 30 – 60 µg/ml blood for human leukocytes.

<sup>b</sup> 1 – 5 µg/ml blood for human leukocytes.

<sup>c</sup> Average size of mRNA molecule = 1930 bases.

### RNA content of cells in culture

Type of cell	Total RNA (mRNA (µg/10 <sup>7</sup> cells))	mRNA (µg/10 <sup>7</sup> cells)
NIH/3T3 cells	75 – 200	1.5 – 4.0
HeLa cells	100 – 300	2 – 6
CHO cells	200 – 400	3 – 6

## C. Ordering Information

<b>Isolation of Nucleic Acids</b>		
<b>Kits to Purify DNA</b>		
Agarose Gel DNA Extraction Kit	1 kit (for up to 10 preparations)	11 696 505 001
DNA Isolation Kit for Cells and Tissues	1 kit (buffer for 20 maxi preps or 60 midi preps)	11 814 770 001
DNA Isolation Kit for Mammalian Blood	1 kit (25 purifications of 10 ml samples)	11 667 327 001
Genopure Plasmid Midi Kit	1 kit (for up to 20 preparations)	03 143 414 001
Genopure Plasmid Maxi Kit	1 kit (for up to 10 preparations)	03 143 422 001
Genopure Buffer Set for Low-Copy Number Plasmids	1 set (buffer for 20 maxi preps or 60 midi preps)	04 634 772 001
High Pure 96 UF Cleanup Kit	1 kit (2 × 96 purifications)	04 422 694 001
High Pure 96 UF Cleanup Plates	1 pack (10 × 96 purifications)	04 422 716 001
High Pure PCR Product Purification Kit	1 kit (50 purifications) 1 kit (250 purifications)	11 732 668 001 11 732 676 001
High Pure PCR Template Preparation Kit	1 kit (100 purifications)	11 796 828 001
High Pure Plasmid Isolation Kit	1 kit (50 purifications) 1 kit (250 purifications)	11 754 777 001 11 754 785 001
High Pure Viral Nucleic Acid Kit	1 kit (for up to 100 purifications)	11 858 874 001
High Pure 16 System Viral Nucleic Acid Kit	1 kit (6 × 16 isolations)	12 011 816 001
High Pure Viral Nucleic Acid Buffer Set	1 set (100 isolations)	12 011 875 001
<b>Kits to Purify RNA</b>		
High Pure RNA Isolation Kit	1 kit (50 reactions)	11 828 665 001
High Pure RNA Tissue Kit	1 kit (50 isolations)	12 033 674 001
High Pure RNA Paraffin Kit	1 kit (up to 100 isolations)	03 270 289 001
High Pure Viral RNA Kit	1 kit (100 purifications)	11 858 882 001
High Pure FFPE RNA Micro Kit	1 kit (50 isolations)	04 823 125 001
mRNA Capture Kit	1 kit (192 reactions)	11 787 896 001
mRNA Isolation Kit	1 kit	11 741 985 001
mRNA Isolation Kit for Blood/Bone Marrow	1 kit 30 [100] isolations (from 5 ml [1.5 ml] sample volumes)	11 934 333 001
<b>Single Reagents for Isolation and Purification of Nucleic Acids</b>		
Streptavidin Magnetic Particles	2 ml 10 ml	11 641 778 001 11 641 786 001
RNA/DNA Stabilization Reagent for Blood/Bone Marrow	500 ml	11 934 317 001
Red Blood Cell Lysis Buffer	100 ml	11 814 389 001
TriPure Isolation Reagent	50 ml 200 ml	11 667 157 001 11 667 165 001
Cesium Chloride (CsCl)	1 kg	10 757 306 001
Phenol	500 g	11 814 303 001
<b>Enzymes to Degrade Cellular Components</b>		
Proteinase K, recombinant, PCR Grade, lyophilizate	25 mg 100 mg 2 × 250 mg 4 × 250 mg	03 115 836 001 03 115 879 001 03 115 801 001 03 115 852 001
Proteinase K, recombinant, PCR Grade, solution	1.25 ml 5 ml 25 ml	03 115 887 001 03 115 828 001 03 115 844 001



**Isolation of Nucleic Acids****Deoxyribonucleases**

DNase I recombinant	2 × 10,000 U	04 536 282 001
DNase I	100 mg	10 104 159 001
DNase I recombinant, RNase-free	10,000 U (provided with incubation buffer)	04 716 728 001

**Ribonucleases**

RNase	500 mg	10 109 134 001
RNase, DNase-free	500 µg (1 ml)	11 119 915 001
RNase A	25 mg	10 109 142 001
	100 mg	10 109 169 001
RNase H	25 U	10 786 349 001
	100 U	10 786 357 001
RNase T1	100,000 U	10 109 193 001

**Other Enzymes**

Lysozyme	10 g	10 837 059 001
Pronase	1 g (non-sterile)	10 165 921 001
	5 g (non-sterile)	11 459 643 001

**Additional Reagents**

Bovine Serum Albumin	20 mg (1 ml)	10 711 454 001
Formamide	500 ml	11 814 320 001

**Amplification of Nucleic Acids****Enzymes for PCR**

Taq DNA Polymerase, 5 U/µl	100 U	11 146 165 001
	500 U (2 × 250 U)	11 146 173 001
	1,000 U (4 × 250 U)	11 418 432 001
	2,500 U (10 × 250 U)	11 596 594 001
	5,000 U (20 × 250 U)	11 435 094 001
Taq DNA Polymerase, 1 U/µl	250 U	11 647 679 001
	1,000 U (4 × 250 U)	11 647 687 001
Taq DNA Polymerase, GMP Grade	1,000 U	03 734 927 001
	5,000 U	03 734 935 001
FastStart Taq DNA Polymerase, 5 U/µl	50 U	12 158 264 001
	100 U	12 032 902 001
	500 U (2 × 250 U)	12 032 929 001
	1,000 U (4 × 250 U)	12 032 937 001
	2,500 U (10 × 250 U)	12 032 945 001
FastStart High Fidelity PCR System	5,000 U (20 × 250 U)	12 032 953 001
	125 U	03 553 426 001
	500 U (2 × 250 U)	03 553 400 001
Expand High Fidelity <sup>PLUS</sup> PCR System	2,500 U (10 × 250 U)	03 553 361 001
	125 U	03 300 242 001
	500 U (2 × 250 U)	03 300 226 001
Expand High Fidelity PCR System	2,500 U (10 × 250 U)	03 300 234 001
	100 U	11 732 641 001
	500 U (2 × 250 U)	11 732 650 001
Expand 20 kb <sup>PLUS</sup> PCR System	2,500 U (10 × 250 U)	11 759 078 001
	200 U	11 811 002 001
GC-RICH PCR System	100 U	12 140 306 001

<b>Amplification of Nucleic Acids</b>		
<b>Enzymes for PCR</b>		
Pwo DNA Polymerase*	100 U	11 644 947 001
	500 U (2 × 250 U)	11 644 955 001
Pwo SuperYield DNA Polymerase*	100 U	04 340 868 001
	500 U (2 × 250 U)	04 340 850 001
<b>Enzyme dNTPacks for PCR</b>		
Taq DNA Polymerase, dNTPack	100 U	04 728 866 001
	500 U (2 × 250 U)	04 728 874 001
	1,000 U (4 × 250 U)	04 728 882 001
	2,500 U (10 × 250 U)	04 728 904 001
	5,000 U (20 × 250 U)	04 728 858 001
Taq DNA Polymerase (1 U/μl), dNTPack	250 U	04 738 225 001
	1,000 U (4 × 250 U)	04 738 241 001
FastStart Taq DNA Polymerase, dNTPack	100 U	04 738 314 001
	500 U (2 × 250 U)	04 738 357 001
	1,000 U (4 × 250 U)	04 738 381 001
	2,500 U (10 × 250 U)	04 738 403 001
	5,000 U (20 × 250 U)	04 738 420 001
FastStart High Fidelity PCR System, dNTPack	125 U	04 738 284 001
	500 U (2 × 250 U)	04 738 292 001
	2,500 U (10 × 250 U)	04 738 306 001
Expand High Fidelity <sup>PLUS</sup> PCR System, dNTPack	125 U	04 743 725 001
	500 U (2 × 250 U)	04 743 733 001
	2,500 U (10 × 250 U)	04 743 374 001
Expand High Fidelity PCR System, dNTPack	100 U	04 738 250 001
	500 U (2 × 250 U)	04 738 268 001
	2,500 U (10 × 250 U)	04 738 276 001
Expand Long Range, dNTPack	50 Reactions	04 829 034 001
	200 Reactions	04 829 042 001
	1000 Reactions	04 829 069 001
Expand 20 kb <sup>PLUS</sup> PCR System, dNTPack	200 U	04 743 814 001
GC-RICH PCR System, dNTPack	100 U	04 743 784 001
Pwo SuperYield DNA Polymerase, dNTPack*	100 U	04 743 750 001
	500 U (2 × 250 U)	04 743 776 001
<b>Master Mixes and Kits for PCR with Polymerase</b>		
PCR Master	1 kit (for 200 reactions of 50 μl final reaction volume)	11 636 103 001
FastStart PCR Master	2 × 1.25 ml (for 100 reactions of 50 μl final reaction volume)	04 710 436 001
	8 × 1.25 ml (for 400 reactions of 50 μl final reaction volume)	04 710 444 001
	10 × 5 ml (for 2,000 reactions of 50 μl final reaction volume)	04 710 452 001
High Fidelity PCR Master	1 kit (for 200 reactions of 50 μl final reaction volume)	12 140 314 001
Pwo Master*	2.5 ml (10 × 250 μl) (for 100 reactions of 50 μl final reaction volume)	03 789 403 001
PCR Core Kit	1 kit (for 100 reactions of 50 μl final reaction volume)	11 578 553 001
PCR Core Kit <sup>PLUS</sup>	1 kit (for 50 reactions of 50 μl final reaction volume)	11 585 541 001
DOP PCR Master	1 kit (for 60 reactions of 50 μl final reaction volume)	11 644 963 001



### Amplification of Nucleic Acids

#### Master Mixes for Real-Time PCR

FastStart TaqMan <sup>®</sup> Probe Master (Rox)	2 × 1.25 ml (for 100 reactions of 50 µl final reaction volume)	04 673 450 001
	10 × 1.25 ml (for 500 reactions of 50 µl final reaction volume)	04 673 468 001
	10 × 5 ml (for 2,000 reactions of 50 µl final reaction volume)	04 673 476 001
FastStart TaqMan <sup>®</sup> Probe Master	2 × 1.25 ml (for 100 reactions of 50 µl final reaction volume)	04 673 409 001
	10 × 1.25 ml (for 500 reactions of 50 µl final reaction volume)	04 673 417 001
	10 × 5 ml (for 2,000 reactions of 50 µl final reaction volume)	04 673 433 001
FastStart SYBR Green Master (Rox)	5 ml (4 × 1.25 ml) (for 200 reactions of 50 µl final reaction volume)	04 673 514 001
	50 ml (10 × 5 ml) (for 2,000 reactions of 50 µl final reaction volume)	04 673 522 001
FastStart SYBR Green Master	5 ml (4 × 1.25 ml) (for 200 reactions of 50 µl final reaction volume)	04 673 484 001
	50 ml (10 × 5 ml) (for 2,000 reactions of 50 µl final reaction volume)	04 673 492 001
ROX Reference Dye	50 µl (1 mM)	04 673 549 001

#### Products for Two-Step RT-PCR

Transcriptor Reverse Transcriptase	250 U (25 reactions)	03 531 317 001
	500 U (50 reactions)	03 531 295 001
	2,000 U (4 × 500 U) (200 reactions)	03 531 287 001
Transcriptor First Strand cDNA Synthesis Kit	1 kit (50 reactions)	04 379 012 001
	1 kit (100 reactions)	04 896 866 001
	1 kit (200 reactions)	04 897 030 001
First Strand cDNA Synthesis Kit for RT-PCR (AMV)	1 kit (30 reactions)	11 483 188 001
cDNA Synthesis System	1 kit (for 10 reactions)	11 117 831 001
Expand Reverse Transcriptase*	1,000 U (20 µl)	11 785 826 001
	5,000 U (100 µl)	11 785 834 001
Reverse Transcriptase M-MuLV	500 U	11 062 603 001
Reverse Transcriptase AMV	500 U	11 495 062 001
	1,000 U	10 109 118 001
5'/3' RACE Kit, 2 <sup>nd</sup> Generation	1 kit (10 reactions)	03 353 621 001

#### Additional Reagents

Protector RNase Inhibitor	2,000 U	03 335 399 001
	10,000 U (5 × 2,000 U)	03 335 402 001
RNase H	25 U	10 786 349 001
	100 U	10 786 357 001
RNA	100 g	10 109 223 001
RNA, MS2	500 µl (10 A <sub>260</sub> units)	10 165 948 001
RNA, 16S- and 23S-ribosomal	1 ml (100 A <sub>260</sub> units)	10 206 938 001

<b>Amplification of Nucleic Acids</b>		
<b>Products for One-Step RT-PCR</b>		
Titan One Tube RT-PCR System	25 reactions	11 888 382 001
	100 reactions	11 855 476 001
Titan One Tube RT-PCR Kit	50 reactions, including 10 control reactions	11 939 823 001
C.therm. Polymerase One-Step RT-PCR System	50 reactions	12 016 338 001
	250 reactions	12 016 346 001
Tth DNA Polymerase	100 U	11 480 014 001
	2 × 250 U	11 480 022 001
Protector RNase Inhibitor	100 U	11 480 014 001
	2 × 250 U	11 480 022 001
<b>PCR-Grade Nucleotides</b>		
PCR Nucleotide Mix	200 µl (for 200 reactions of 50 µl final reaction volume)	11 581 295 001
	2,000 µl (10 × 200 µl) (for 2,000 reactions of 50 µl final reaction volume)	11 814 362 001
PCR Nucleotide Mix <sup>PLUS</sup>	200 µl (2 × 100 µl) (for 200 reactions of 50 µl final reaction volume)	11 888 412 001
Deoxynucleoside Triphosphate Set	4 × 250 µl (25 µmol) (for 2,500 reactions at 50 µl final volume)	11 969 064 001
	4 × 1,250 µl (125 µmol) (for 12,500 reactions at 50 µl final volume)	03 622 614 001
dATP	250 µl (25 µmol)	11 934 511 001
	1,250 µl (125 µmol)	11 969 013 001
	4 × 1,250 µl (4 × 125 µmol)	03 732 681 001
dCTP	250 µl (25 µmol)	11 934 520 001
	1,250 µl (125 µmol)	11 969 021 001
	4 × 1,250 µl (4 × 125 µmol)	03 732 690 001
dGTP	250 µl (25 µmol)	11 934 538 001
	1,250 µl (125 µmol)	11 969 030 001
	4 × 1,250 µl (4 × 125 µmol)	03 732 703 001
dTTP	25 µl (25 µmol)	11 934 546 001
	1,250 µl (125 µmol)	11 969 048 001
	4 × 1,250 µl (4 × 125 µmol)	03 732 711 001
dUTP	250 µl (25 µmol)	11 934 554 001
	1,250 µl (125 µmol)	11 969 056 001
	4 × 1,250 µl (4 × 125 µmol)	03 732 720 001
<b>Optimization of Standard PCR</b>		
PCR Optimization Kit	1 kit	11 636 138 001
Uracil-DNA Glycosylase	100 U	11 444 646 001
Uracil-DNA Glycosylase, heat-labile	100 U	11 775 367 001
	500 U	11 775 375 001
T4 Gene 32 Protein	100 µg	10 972 983 001
	500 µg	10 972 991 001
Tth Pyrophosphatase, thermostable	100 U	11 721 992 001
PCR Buffer Set	2 × 2 ml (2 × 1 ml of each solution)	11 699 121 001
PCR Buffer Without MgCl <sub>2</sub> , 10× conc.	3 × 1 ml	11 699 105 001
MgCl <sub>2</sub> Stock Solution	3 × 1 ml	11 699 113 001
Water, PCR Grade	25 ml (25 × 1 ml)	03 315 932 001
	25 ml (1 × 25 ml)	03 315 959 001
	100 ml (4 × 25 ml)	03 315 843 001



### Amplification of Nucleic Acids

#### PCR Cloning Kits

Expand Cloning Kit	1 kit	11 940 392 001
PCR Cloning Kit (blunt end)	1 kit	11 939 645 001
Rapid DNA Ligation Kit	1 kit	11 635 379 001

#### Primer for PCR and RT-PCR Reactions

Primer "random"	2 mg (50 A <sub>260</sub> units, 1 μmol)	11 034 731 001
Primer for cDNA Synthesis	40 μg (1 A <sub>260</sub> unit, 12 nmol)	10 814 261 001
Primer for cDNA Synthesis	40 μg (1 A <sub>260</sub> unit, 8 nmol)	10 814 270 001
Oligo (dT) <sub>20</sub> Probe, Biotin-labeled	2 nmol (20 μl)	11 741 764 001

#### Additional Reagents

Human t-PA Control Primer Set	1 set	11 691 104 001
Human Genomic DNA	100 μg	11 691 112 001
Glycogen	20 mg (1 ml)	10 901 393 001
Streptavidin-Coated PCR Tubes (Strips)	24 strips of 8 × 0.2 ml streptavidin-coated tubes and 24 strips of 8 caps	11 741 772 001
Strip PCR Tubes and Caps	125 strips (8 tubes/strip) 80 strips (12 tubes/strip)	11 667 009 001 11 667 017 001

#### Buffers

Buffers in a Box, Premixed PBS Buffer, 10×	4 l	11 666 789 001
Buffers in a Box, Premixed TAE Buffer, 10×	4 l	11 666 690 001
Buffers in a Box, Premixed TBE Buffer, 10×	4 l	11 666 703 001



Mapping and Cloning of Nucleic Acids		
Restriction Enzymes		
<i>Aat</i> II	250 U (1 - 5 U/ $\mu$ l)	10 775 207 001
<i>Acc</i> I	100 U (5 U/ $\mu$ l) 500 U (5 U/ $\mu$ l)	10 728 420 001 10 728 438 001
<i>Acs</i> I	200 U (10 U/ $\mu$ l)	11 526 456 001
<i>Acy</i> I ( <i>Aha</i> II)	200 U (5 U/ $\mu$ l)	11 081 314 001
<i>Aff</i> III	100 U (5 U/ $\mu$ l)	11 209 183 001
<i>Alu</i> I	500 U (10 U/ $\mu$ l) 2,000 U (10 U/ $\mu$ l)	10 239 275 001 10 656 267 001
<i>Alw</i> 44 I ( <i>Sno</i> I)	1,000 U (10 U/ $\mu$ l)	11 450 506 001
<i>Apa</i> I	5,000 U (10 U/ $\mu$ l) 5,000 U (40 U/ $\mu$ l) 20,000 U (40 U/ $\mu$ l)	10 899 208 001 10 703 745 001 10 703 753 001
<i>Asp</i> I ( <i>Tth</i> 111 I)	400 U (10 U/ $\mu$ l)	11 131 354 001
<i>Asp</i> 700 ( <i>Xmn</i> I)	500 U (10 U/ $\mu$ l)	10 835 277 001
<i>Asp</i> 718	1,000 U (10 U/ $\mu$ l) 5,000 U (10 U/ $\mu$ l) 5,000 U (40 U/ $\mu$ l)	10 814 245 001 10 814 253 001 11 175 050 001
<i>Asp</i> EI	200 U (10 U/ $\mu$ l)	11 428 179 001
<i>Ava</i> I	1,000 U (5 U/ $\mu$ l)	10 740 730 001
<i>Ava</i> II	500 U (5 U/ $\mu$ l)	10 740 756 001
<i>Avi</i> II ( <i>Aos</i> I)	200 U (10 U/ $\mu$ l)	11 481 436 001
<i>Bam</i> HI	1,000 U (10 U/ $\mu$ l) 2,500 U (10 U/ $\mu$ l) 10,000 U (10 U/ $\mu$ l) 10,000 U (40 U/ $\mu$ l) 50,000 U (40 U/ $\mu$ l)	10 220 612 001 10 567 604 001 10 656 275 001 10 798 975 001 11 274 031 001
<i>Ban</i> II	500 U (10 U/ $\mu$ l)	10 775 240 001
<i>Bbr</i> PI ( <i>Pma</i> CI)	500 U (10 U/ $\mu$ l)	11 168 860 001
<i>Bcl</i> I	500 U (10 U/ $\mu$ l) 2,500 U (40 U/ $\mu$ l)	10 693 952 001 11 097 059 001
<i>Bfr</i> I ( <i>Aff</i> II)	500 U (10 U/ $\mu$ l)	11 198 939 001
<i>Bgl</i> I	1,000 U (10 U/ $\mu$ l) 5,000 U (40 U/ $\mu$ l)	10 404 101 001 11 047 604 001
<i>Bgl</i> II	500 U (10 U/ $\mu$ l) 2,000 U (10 U/ $\mu$ l) 2,000 U (40 U/ $\mu$ l) 10,000 U (40 U/ $\mu$ l)	10 348 767 001 10 567 639 001 10 899 224 001 11 175 068 001
<i>Bln</i> I ( <i>Avr</i> II)	200 U (10 U/ $\mu$ l) 1,000 U (10 U/ $\mu$ l)	11 558 161 001 11 558 170 001
<i>Bpu</i> AI	200 U (10 U/ $\mu$ l)	11 497 944 001
<i>Bse</i> AI	200 U (10 U/ $\mu$ l)	11 417 169 001
<i>Bsi</i> WI	300 U (10 U/ $\mu$ l)	11 388 959 001
<i>Bsi</i> YI	200 U (10 U/ $\mu$ l)	11 388 916 001
<i>Bsm</i> I	200 U (10 U/ $\mu$ l)	11 292 307 001
<i>Bsp</i> LU11 I	200 U (10 U/ $\mu$ l)	11 693 743 001
<i>Bss</i> HII	200 U (10 U/ $\mu$ l)	11 168 851 001
<i>Bst</i> 1107 I	200 U (10 U/ $\mu$ l)	11 378 953 001
<i>Bst</i> EII	500 U (10 U/ $\mu$ l)	10 404 233 001
<i>Bst</i> XI	250 U (10 U/ $\mu$ l) 1,250 U (10 U/ $\mu$ l)	11 117 777 001 11 117 785 001



## Mapping and Cloning of Nucleic Acids

## Restriction Enzymes

<i>Cel</i> II	200 U (10 U/ $\mu$ l)	11 449 397 001
<i>Cfo</i> I ( <i>Hha</i> I)	1,000 U (10 U/ $\mu$ l)	10 688 541 001
	5,000 U (10 U/ $\mu$ l)	10 688 550 001
<i>Cla</i> I	500 U (10 U/ $\mu$ l)	10 404 217 001
	2,500 U (10 U/ $\mu$ l)	10 656 291 001
	2,500 U (40 U/ $\mu$ l)	11 092 758 001
<i>Dde</i> I	1,000 U (10 U/ $\mu$ l)	10 835 307 001
<i>Dpn</i> I	200 U (10 U/ $\mu$ l)	10 742 970 001
	1,000 U (10 U/ $\mu$ l)	10 742 988 001
<i>Dra</i> I	5,000 U (10 U/ $\mu$ l)	10 827 754 001
	10,000 U (40 U/ $\mu$ l)	11 175 076 001
<i>Dra</i> II	250 U (1 - 3 U/ $\mu$ l)	10 843 512 001
<i>Dra</i> III	500 U (1 - 5 U/ $\mu$ l)	10 843 547 001
<i>Eae</i> I ( <i>Cfr</i> I)	200 U (10 U/ $\mu$ l)	11 062 557 001
<i>Ecl</i> XI ( <i>Xma</i> III)	1,000 U (10 U/ $\mu$ l)	11 131 397 001
<i>Eco</i> 47 III	100 U (5 U/ $\mu$ l)	11 167 103 001
<i>Eco</i> RI	5,000 U (10 U/ $\mu$ l)	10 703 737 001
	10,000 U (10 U/ $\mu$ l)	11 175 084 001
	10,000 U (40 U/ $\mu$ l)	10 200 310 001
	50,000 U (40 U/ $\mu$ l)	10 606 189 001
<i>Eco</i> RII ( <i>Bst</i> NI)	200 U (10 U/ $\mu$ l)	11 427 881 001
<i>Eco</i> RV	2,000 U (10 U/ $\mu$ l)	10 667 145 001
	10,000 U (10 U/ $\mu$ l)	10 667 153 001
	10,000 U (40 U/ $\mu$ l)	11 040 197 001
<i>Fok</i> I	100 U (1 - 5 U/ $\mu$ l)	11 004 816 001
<i>Hae</i> II	100 U (5 U/ $\mu$ l)	10 693 910 001
<i>Hae</i> III	5,000 U (10 U/ $\mu$ l)	10 693 944 001
<i>Hind</i> II	2,500 U (3 - 10 U/ $\mu$ l)	10 656 305 001
<i>Hind</i> III	5,000 U (10 U/ $\mu$ l)	10 656 313 001
	10,000 U (10 U/ $\mu$ l)	10 656 321 001
	10,000 U (40 U/ $\mu$ l)	10 798 983 001
	50,000 U (40 U/ $\mu$ l)	11 274 040 001
<i>Hinf</i> I	1,000 U (10 U/ $\mu$ l)	10 779 652 001
	5,000 U (10 U/ $\mu$ l)	10 779 679 001
	20,000 U (40 U/ $\mu$ l)	11 274 082 001
<i>Hpa</i> I	100 U (3 - 10 U/ $\mu$ l)	10 380 385 001
	500 U (3 - 10 U/ $\mu$ l)	10 567 647 001
<i>Hpa</i> II	5,000 U (10 U/ $\mu$ l)	10 656 330 001
	5,000 U (40 U/ $\mu$ l)	11 207 598 001
<i>Ita</i> I 17	200 U (10 U/ $\mu$ l)	11 497 979 001
<i>Kpn</i> I	5,000 U (10 U/ $\mu$ l)	10 899 186 001
	10,000 U (40 U/ $\mu$ l)	10 742 953 001
<i>Ksp</i> I ( <i>Sac</i> II)	1,000 U (10 U/ $\mu$ l)	11 117 807 001
<i>Ksp</i> 632 I	200 U (10 U/ $\mu$ l)	11 081 276 001
<i>Mae</i> I	50 U (1 - 5 U/ $\mu$ l)	10 822 213 001
	250 U (1 - 5 U/ $\mu$ l)	10 822 221 001
<i>Mae</i> II	50 U (1 - 5 U/ $\mu$ l)	10 862 495 001
<i>Mae</i> III	50 U (1 - 5 U/ $\mu$ l)	10 822 230 001
	250 U (1 - 5 U/ $\mu$ l)	10 822 248 001
<i>Mam</i> I	200 U (10 U/ $\mu$ l)	11 131 281 001

Mapping and Cloning of Nucleic Acids		
Restriction Enzymes		
<i>Mlu</i> I	500 U (10 U/ $\mu$ l)	10 909 700 001
	2,500 U (10 U/ $\mu$ l)	10 909 718 001
	2,500 U (40 U/ $\mu$ l)	11 207 601 001
<i>Mlu</i> NI ( <i>Bal</i> I)	200 U (10 U/ $\mu$ l)	11 526 430 001
<i>Mro</i> I ( <i>Acc</i> III)	100 U ( $\geq$ 1 - 5 U/ $\mu$ l)	11 102 982 001
<i>Msp</i> I	5,000 U (10 U/ $\mu$ l)	10 633 526 001
	5,000 U (40 U/ $\mu$ l)	11 047 647 001
<i>Mun</i> I ( <i>Mfe</i> I)	200 U (10 U/ $\mu$ l)	11 441 337 001
<i>Mva</i> I ( <i>Bst</i> NI)	5,000 U (10 U/ $\mu$ l)	11 288 075 001
<i>Mvn</i> I ( <i>Fnu</i> DII)	200 U (10 U/ $\mu$ l)	11 062 573 001
<i>Nae</i> I	1,000 U (10 U/ $\mu$ l)	10 786 322 001
<i>Nar</i> I	200 U (10 U/ $\mu$ l)	11 103 016 001
	1,000 U (10 U/ $\mu$ l)	11 103 024 001
<i>Nco</i> I	200 U (10 U/ $\mu$ l)	10 835 315 001
	1,000 U (10 U/ $\mu$ l)	10 835 323 001
	1,000 U (40 U/ $\mu$ l)	11 047 698 001
<i>Nde</i> I	200 U (10 U/ $\mu$ l)	11 040 219 001
	1,000 U (10 U/ $\mu$ l)	11 040 227 001
<i>Nde</i> II ( <i>Mbo</i> I)	1,000 U (5 U/ $\mu$ l)	11 040 243 001
<i>Nhe</i> I	200 U (10 U/ $\mu$ l)	10 885 843 001
	1,000 U (10 U/ $\mu$ l)	10 885 851 001
	1,500 U (40 U/ $\mu$ l)	10 885 860 001
<i>Not</i> I	200 U (10 U/ $\mu$ l)	11 014 706 001
	1,000 U (10 U/ $\mu$ l)	11 014 714 001
	1,000 U (40 U/ $\mu$ l)	11 037 668 001
<i>Nru</i> I	200 U (10 U/ $\mu$ l)	10 776 769 001
	1,000 U (10 U/ $\mu$ l)	10 776 777 001
<i>Nsi</i> I	200 U (10 U/ $\mu$ l)	10 909 831 001
	1,000 U (10 U/ $\mu$ l)	10 909 840 001
<i>Nsp</i> I	200 U (10 U/ $\mu$ l)	11 131 419 001
<i>Pin</i> AI ( <i>Age</i> I)	200 U (10 U/ $\mu$ l)	11 464 841 001
	1,000 U (10 U/ $\mu$ l)	11 464 850 001
<i>Pst</i> I	3,000 U (10 U/ $\mu$ l)	10 621 625 001
	10,000 U (10 U/ $\mu$ l)	10 621 633 001
	10,000 U (40 U/ $\mu$ l)	10 798 991 001
<i>Pvu</i> I	100 U (5 U/ $\mu$ l)	10 650 137 001
	500 U (5 U/ $\mu$ l)	10 650 129 001
<i>Pvu</i> II	1,000 U (10 U/ $\mu$ l)	10 642 690 001
	5,000 U (10 U/ $\mu$ l)	10 642 703 001
<i>Rca</i> I ( <i>Bsp</i> HI)	200 U (5 U/ $\mu$ l)	11 467 123 001
<i>Rsa</i> I	1,000 U (10 U/ $\mu$ l)	10 729 124 001
	5,000 U (10 U/ $\mu$ l)	10 729 132 001
	5,000 U (40 U/ $\mu$ l)	11 047 671 001
<i>Rsr</i> II	200 U (10 U/ $\mu$ l)	11 292 587 001
<i>Sac</i> I ( <i>Sst</i> I)	1,000 U (10 U/ $\mu$ l)	10 669 792 001
	5,000 U (10 U/ $\mu$ l)	10 669 806 001
	5,000 U (40 U/ $\mu$ l)	11 047 655 001
<i>Sal</i> I	500 U (10 U/ $\mu$ l)	10 348 783 001
	2,500 U (10 U/ $\mu$ l)	10 567 663 001
	2,500 U (40 U/ $\mu$ l)	11 047 612 001
<i>Sau</i> 3AI	500 U (1 - 5 U/ $\mu$ l)	10 709 751 001
<i>Sau</i> 96 I	300 U (10 U/ $\mu$ l)	10 651 303 001



## Mapping and Cloning of Nucleic Acids

### Restriction Enzymes

<i>Sca</i> I	500 U (10 U/ $\mu$ l)	10 775 258 001
	2,500 U (10 U/ $\mu$ l)	10 775 266 001
	5,000 U (40 U/ $\mu$ l)	11 207 636 001
<i>Scr</i> FI ( <i>Dsa</i> V)	500 U (10 U/ $\mu$ l)	11 081 292 001
<i>Sex</i> AI	200 U (10 U/ $\mu$ l)	11 497 995 001
<i>Sfi</i> I	1,250 U (10 U/ $\mu$ l)	11 288 024 001
	5,000 U (40 U/ $\mu$ l)	11 288 059 001
<i>Sfu</i> I ( <i>Asu</i> II)	2,000 U (10 U/ $\mu$ l)	11 243 497 001
<i>Sgr</i> AI	200 U (10 U/ $\mu$ l)	11 277 014 001
<i>Sma</i> I	1,000 U (10 U/ $\mu$ l)	10 220 566 001
	5,000 U (10 U/ $\mu$ l)	10 656 348 001
	5,000 U (40 U/ $\mu$ l)	11 047 639 001
<i>Sna</i> BI	200 U (10 U/ $\mu$ l)	10 997 480 001
<i>Spe</i> I	200 U (10 U/ $\mu$ l)	11 008 943 001
	1,000 U (10 U/ $\mu$ l)	11 008 951 001
	1,000 U (40 U/ $\mu$ l)	11 207 644 001
<i>Sph</i> I	200 U (10 U/ $\mu$ l)	11 026 950 001
	500 U (10 U/ $\mu$ l)	10 606 120 001
	2,500 U (10 U/ $\mu$ l)	11 026 534 001
	2,500 U (40 U/ $\mu$ l)	11 026 542 001
<i>Ssp</i> I	1,000 U (10 U/ $\mu$ l)	10 972 975 001
	1,000 U (40 U/ $\mu$ l)	11 207 652 001
<i>Ssp</i> BI	200 U € 50,50 (10 U/ $\mu$ l)	11 497 901 001
<i>Stu</i> I	500 U (10 U/ $\mu$ l)	10 753 351 001
	2,500 U (10 U/ $\mu$ l)	10 753 360 001
	2,500 U (40 U/ $\mu$ l)	11 047 680 001
<i>Sty</i> I	2,000 U (10 U/ $\mu$ l)	11 047 744 001
<i>Swa</i> I	200 U (10 U/ $\mu$ l)	11 371 517 001
	1,000 U (10 U/ $\mu$ l)	11 371 525 001
<i>Taq</i> I	2,500 U (10 U/ $\mu$ l)	10 567 671 001
	10,000 U (10 U/ $\mu$ l)	11 175 114 001
<i>Tru</i> 9 I	1,000 U (10 U/ $\mu$ l)	11 464 825 001
<i>Van</i> 91 I ( <i>Pfl</i> MI)	200 U (5 U/ $\mu$ l)	11 379 275 001
<i>Xba</i> I	1,000 U (10 U/ $\mu$ l)	10 674 257 001
	5,000 U (10 U/ $\mu$ l)	10 674 265 001
	20,000 U (10 U/ $\mu$ l)	10 674 273 001
	20,000 U (40 U/ $\mu$ l)	11 047 663 001
<i>Xho</i> I	2,500 U (40 U/ $\mu$ l)	10 703 770 001
	5,000 U (10 U/ $\mu$ l)	10 899 194 001
	12,500 U (40 U/ $\mu$ l)	10 703 788 001
<i>Xho</i> II	50 U (1 - 5 U/ $\mu$ l)	10 742 929 001
<i>Xma</i> CI	200 U (10 U/ $\mu$ l)	11 743 392 001

### SuRE/Cut Buffers for Restriction Enzymes

SuRE/Cut Buffer Set for Restriction Enzymes	1 set	11 082 035 001
SuRE/Cut Buffer A	5 × 1 ml	11 417 959 001
SuRE/Cut Buffer B	5 × 1 ml	11 417 967 001
SuRE/Cut Buffer H	5 × 1 ml	11 417 991 001
SuRE/Cut Buffer L	5 × 1 ml	11 417 975 001
SuRE/Cut Buffer M	5 × 1 ml	11 417 983 001

<b>Nucleases</b>		
<b>Nucleases</b>		
DNase I recombinant, grade I	2 × 10,000 U	04 536282 001
DNase I, RNase-free	10,000 U	04 716 728 001
Nuclease S1	50,000 U	10 818 348 001
Nuclease S7	15,000 U	10 107 921 001
<b>Ribonucleases</b>		
RNase	500 mg	10 109 134 001
RNase, DNase-free	500 µg (1 ml)	11 119 915 001
RNase A	25 mg	10 109 142 001
	100 mg	10 109 169 001
RNase H	25 mg	10 786 349 001
	100 mg	10 786 357 001
RNaseT1	100,000 U	10 109 193 001
<b>Cloning of DNA Fragments</b>		
<b>Ligases and Phosphatases</b>		
T4 DNA Ligase	100 U (1 U/µl)	10 481 220 001
	500 U (1 U/µl)	10 716 359 001
	500 U (5 U/µl)	10 799 009 001
Alkaline Phosphatase, shrimp	1,000 U	11 758 250 001
Alkaline Phosphatase	1,000 U (20 U/µl)	11 097 075 001
	1,000 U (1 U/µl)	10 713 023 001
T4 RNA Ligase	500 U	11 449 478 001
<b>Modifying Enzymes</b>		
Klenow Enzyme (labeling grade, DNA Polymerase I, large fragment)	100 U	11 008 404 001
	500 U	11 008 412 001
T4 DNA Polymerase	100 U	11 004 786 001
	500 U	11 004 794 001
T7 RNA Polymerase	1,000 U	10 881 767 001
	5,000 U	10 881 775 001
<b>DNA Molecular Weight Markers</b>		
DNA Molecular Weight Marker II	50 µg (1 A <sub>260</sub> unit)	10 236 250 001
DNA Molecular Weight Marker III	50 µg (1 A <sub>260</sub> unit)	10 528 552 001
DNA Molecular Weight Marker IV	50 µg (1 A <sub>260</sub> unit)	11 418 009 001
DNA Molecular Weight Marker V	50 µg (1 A <sub>260</sub> unit)	10 821 705 001
DNA Molecular Weight Marker VI	50 µg (1 A <sub>260</sub> unit)	11 062 590 001
DNA Molecular Weight Marker VII	50 µg (1 A <sub>260</sub> unit)	11 209 264 001
DNA Molecular Weight Marker VIII	50 µg (1 A <sub>260</sub> unit)	11 336 045 001
DNA Molecular Weight Marker IX	50 µg (1 A <sub>260</sub> unit)	11 449 460 001
DNA Molecular Weight Marker X	100 µg (2 A <sub>260</sub> units)	11 498 037 001
DNA Molecular Weight Marker XIII (50 bp ladder)	50 µg (1 A <sub>260</sub> unit)	11 721 925 001
DNA Molecular Weight Marker XIV (100 bp ladder)	50 µg (1 A <sub>260</sub> unit)	11 721 933 001
DNA Molecular Weight Marker XV (Expand DNA Molecular Weight Marker)	50 µg (1 A <sub>260</sub> unit)	11 721 615 001
DNA Molecular Weight Marker XVI (250 bp ladder)	50 µg (200 µl)	11 855 638 001
DNA Molecular Weight Marker XVII (500 bp ladder)	50 µg (200 µl)	11 855 646 001



**Cloning of DNA Fragments****Agaroses and Dyes**

Aarose LE	100 g	11 685 660 001
	500 g	11 685 678 001
Agarose MP	100 g	11 388 983 001
	500 g	11 388 991 001
Agarose MS	100 g	11 816 586 001
	500 g	11 816 594 001
SYBR Green I Nucleic Acid Gel Stain	0.5 ml	11 988 131 001

**Nucleotides****Deoxynucleotides: Mixes, Sets, and Single Nucleotides**

Deoxynucleoside Triphosphate Set	4 × 10 μmol (4 × 100 μl)	11 277 049 001
	40 × 10 μmol (40 × 100 μl)	11 922 505 001
dATP	250 μl (25 μmol)	11 051 440 001
dCTP	250 μl (25 μmol)	11 051 458 001
dGTP	250 μl (25 μmol)	11 051 466 001
dTTP	250 μl (25 μmol)	11 051 482 001
dUTP	250 μl (25 μmol)	11 420 470 001
dITP	25 μmol (250 μl)	11 051 474 001

**Modified Nucleotides**

7-Deaza-2'-deoxy-guanosine-5'-triphosphate	2 μmol (200 μl)	10 988 537 001
5'-Methyl-2'-deoxycytidine-5'-triphosphate	10 μmol	10 757 047 001

**Dideoxynucleotides**

Dideoxynucleoside Triphosphate Set	4 × 1 μmol (4 × 100 μl)	03 732 738 001
Dideoxynucleoside Triphosphate Set	4 × 1 μmol (4 × 100 μl)	11 008 382 001

**Ribonucleotides**

ADP	1 g	10 127 507 001
Ribonucleoside Triphosphate Set	4 × 20 μmol	11 277 057 001
ATP	5 g	10 127 523 001
	10 g	10 127 531 001
ATP	1 g	10 519 979 001
	5 g	10 519 987 001
ATP	40 μmol (400 μl)	11 140 965 001
CTP	500 mg	10 103 845 001
CTP	40 μmol (400 μl)	11 140 922 001
GTP	250 mg	10 106 399 001
GTP	40 μmol (400 μl)	11 140 957 001
UTP	500 mg	10 110 221 001
UTP	40 μmol (400 μl)	11 140 949 001

**Polynucleotides**

Poly (A)	100 mg	10 108 626 001
Poly (dA)	5 A <sub>260</sub> units	10 223 581 001
Poly (A) × (dT) <sub>15</sub>	5 A <sub>260</sub> units	10 108 677 001
Poly[d(I-C)]	10 A <sub>260</sub> units	10 108 812 001
	50 A <sub>260</sub> units	11 219 847 001

## Cloning of DNA Fragments

### Nucleotides for Labeling

Digoxigenin-11-dUTP, alkali-labile	25 nmol (25 µl)	11 573 152 910
	125 nmol (125 µl)	11 573 179 910
Digoxigenin-11-dUTP, alkali-stable	25 nmol (25 µl)	11 093 088 910
	125 nmol (125 µl)	11 558 706 910
	5 × 125 nmol (5 × 125 µl)	11 570 013 910
Digoxigenin-11-UTP	250 nmol (10 mM, 25 µl)	11 209 256 910
	200 nmol (3.5 mM, 57 µl)	03 359 247 910
Digoxigenin-11-ddUTP	25 nmol (25 µl)	11 363 905 910
Biotin-16-dUTP	50 nmol (50 µl)	11 093 070 910
Biotin-16-UTP	250 nmol (25 µl)	11 388 908 910
Biotin-16-ddUTP	25 nmol (25 µl)	11 427 598 910
Fluorescein-12-dUTP	25 nmol (25 µl)	11 373 242 910
Fluorescein-12-UTP	250 nmol (25 µl)	11 427 857 910
Tetramethyl-Rhodamine-5-dUTP	25 nmol (25 µl)	11 534 378 910

## Automated Sample Preparation

### MagNA Pure LC

MagNA Pure LC Instrument	1 instrument plus accessories	12 236 931 001
MagNA Pure LC Cooling Block, LC Centrifuge Adapters	1 cooling block with 32 LightCycler® Centrifuge Adapters	12 190 664 001
MagNA Pure LC Cooling Block, LC Sample Carousel	1 cooling block	12 189 704 001
MagNA Pure LC Cooling Block, 96-well PCR Plate	1 cooling block	12 189 674 001

### MagNA Pure LC Kits for DNA Isolation

MagNA Pure LC DNA Isolation Kit I	1 kit (192 isolations)	03 003 990 001
MagNA Pure LC DNA Isolation Kit II (Tissue)	1 kit (192 isolations)	03 186 229 001
MagNA Pure LC DNA Isolation Kit III (Bacteria, Fungi)	1 kit (192 isolations)	03 264 785 001
MagNA Pure LC DNA Isolation Kit - Large Volume	1 kit (96-288 isolations)	03 310 515 001
MagNA Pure LC DNA Isolation Kit - Lysis/Binding Buffer Refill	100 ml	03 246 752 001

### MagNA Pure LC Kits for Total Nucleic Acid Isolation

MagNA Pure LC Total Nucleic Acid Isolation Kit	1 kit (192 reactions)	03 038 505 001
MagNA Pure LC Total Nucleic Acid Isolation Kit - Large Volume	1 kit (192 reactions)	03 264 793 001
MagNA Pure LC Total Nucleic Acid Isolation Kit- Lysis/Binding Buffer Refill	100 ml	03 246 779 001

### MagNA Pure LC Kits for RNA Isolation

MagNA Pure LC RNA Isolation Kit - High Performance	1 kit (192 isolations)	03 542 394 001
MagNA Pure LC RNA Isolation Kit III (Tissue)	1 kit (192 isolations)	03 330 591 001
MagNA Pure LC mRNA Isolation Kit I (Blood, Blood Cells)	1 kit (192 isolations)	03 004 015 001
MagNA Pure LC mRNA HS Kit	1 kit (192 isolations)	03 267 393 001
MagNA Pure LC RNA Isolation Tissue - Lysis Buffer Refill	70 ml	03 604 721 001
MagNA Pure LC mRNA Isolation Kit II (Tissue)	1 kit (192 isolations)	03 172 627 001

### MagNA Pure Compact

MagNA Pure Compact Instrument	1 instrument with integrated PC, touch-screen monitor, and bar-code reader	03 731 146 001
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### Automated Sample Preparation

#### MagNA Pure Compact Kits

MagNA Pure Compact RNA Isolation Kit (Tissue)	1 kit (32 isolations)	04 643 542 001
MagNA Pure Compact Nucleic Acid Isolation Kit I	1 kit (32 isolations)	03 730 964 001
MagNA Pure Compact Nucleic Acid Isolation Kit I -Large Volume	1 kit (32 isolations)	03 730 972 001
MagNA Pure Compact RNA Isolation Kit	1 kit (32 isolations)	04 802 993 001

#### MagNA Lyser

MagNA Lyser Instrument	1 instrument (220 V)	03 358 976 001
	1 instrument (110 V) (plus Rotor and Rotor Cooling Block)	03 358 968 001
MagNA Lyser Green Beads	100 tubes	03 358 941 001

### Real-Time PCR Systems

#### LightCycler® Carousel-Based System

LightCycler® 2.0 Instrument	1 instrument plus accessories	03 351 414 001
LightCycler® 1.5 Instrument	1 instrument plus accessories	04 484 495 001
LC Carousel Centrifuge 2.0	1 centrifuge plus rotor (230 V)	03 709 582 001
	1 centrifuge plus rotor (110 V)	03 709 507 001
LC Carousel Centrifuge 2.0 Rotor Set	1 rotor + 2 buckets	03 724 697 001
LC Carousel Centrifuge 2.0 Bucket 2.1	1 bucket	03 724 689 001
LightCycler® Capillaries (20µl)	1 pack (8 boxes, each with 96 capillaries and stoppers)	11 909 339 001
LightCycler® Probe Design Software 2.0	1 software package	04 342 054 001

#### LightCycler® Carousel-Based System Kits for PCR

LightCycler® DNA Master HybProbe	1 kit (96 reactions)	12 015 102 001
	1 kit (480 reactions)	2 158 825
LightCycler® FastStart DNA Master HybProbe	1 kit (96 reactions)	03 003 248 001
	1 kit (480 reactions)	12 239 272 001
LightCycler® FastStart DNA Master <sup>PLUS</sup> HybProbe	1 kit (96 reactions)	03 515 575 001
	1 kit (480 reactions)	03 515 567 001
LightCycler® FastStart DNA Master <sup>PLUS</sup> HybProbe, 100 µl Reactions	1 kit (384 reactions)	03 752 178 001
LightCycler® DNA Master SYBR Green I	1 kit (96 reactions)	12 015 099 001
	1 kit (480 reactions)	12 158 817 001
LightCycler® FastStart DNA Master SYBR Green I	1 kit (96 reactions)	03 003 230 001
	1 kit (480 reactions)	12 239 264 001
LightCycler® FastStart DNA Master <sup>PLUS</sup> SYBR Green I	1 kit (96 reactions)	03 515 869 001
	1 kit (480 reactions)	03 515 885 001
LightCycler® FastStart DNA Master <sup>PLUS</sup> SYBR Green I, 100 µl Reactions	1 kit (384 reactions)	03 752 186 001

#### Associated Kits and Reagents

LightCycler® Color Compensation Set	1 set (5 reactions)	12 158 850 001
LightCycler® Uracil-DNA Glycosylase	100 U (50 µl)	03 539 806 001
LightCycler® TaqMan Master	1 kit (96 reactions)	04 535 286 001
LightCycler® Multiplex DNA Master HybProbe	1 kit (96 reactions)	04 340 019 001

**Real-Time PCR Systems****LightCycler® 2.0 System Kits for RT-PCR**

LightCycler® RNA Amplification Kit SYBR Green I	1 kit (96 reactions)	12 015 137 001
LightCycler® RNA Amplification Kit HybProbe	1 kit (96 reactions)	12 015 145 001
LightCycler® RNA Master SYBR Green I	1 kit (96 reactions)	03 064 760 001
LightCycler® RNA Master HybProbe	1 kit (96 reactions)	03 018 954 001

**LightCycler® 480 System**

LightCycler® 480 Instrument, 96-well block	1 instrument	04 640 268 001
LightCycler® 480 Instrument, 384-well block	1 instrument	04 545 885 001
LightCycler® 480 Block Kit 96	1 kit	04 643 640 001
LightCycler® 480 Block Kit 384	1 kit	04 643 631 001
LightCycler® 480 Bar-Code Scanner	1 handheld Scanner	04 710 606 001
LightCycler® 480 Basic Software 1.0	CD and manual	04 722 205 001
LightCycler® 480 LIMS/Bar-Code Software	CD and manual	04 727 886 001
LightCycler® 480 Relative Quantification Software	CD and manual	04 727 851 001
LightCycler® 480 Genotyping Software	CD and manual	04 727 860 001
LightCycler® 480 Multiwell Plate, 96	50 plates	04 729 692 001
LightCycler® 480 Multiwell Plate, 384	50 plates	04 729 749 001
LightCycler® 480 Sealing Foil	50 foils	04 729 757 001
LightCycler® 480 SYBR Green I Master	5 × 1 ml	04 707 516 001
LightCycler® 480 Probes Master	5 × 1 ml	04 707 494 001
LightCycler® 480 Genotyping Master	4 × 384 µl	04 707 524 001
LightCycler® 480 Control Kit	3 Control Runs	04 710 924 001

**Universal ProbeLibrary**

Universal ProbeLibrary Set, Human	1 set	04 683 633 001
Universal ProbeLibrary Set, Mouse	1 set	04 683 641 001
Universal ProbeLibrary Set, Rat	1 set	04 683 650 001
Universal ProbeLibrary Set, Primates	1 set	04 683 617 001
Universal ProbeLibrary Set, <i>C. elegans</i>	1 set	04 683 609 001
Universal ProbeLibrary Set, <i>Drosophila</i>	1 set	04 683 625 001
Universal ProbeLibrary Set, <i>Arabidopsis</i>	1 set	04 683 595 001
Universal ProbeLibrary Control Set	1 set	04 696 417 001
Universal ProbeLibrary Extension Set	1 set	04 869 877 001
Single ProbeLibrary Probes, # 1–# 165	500 (50 µl) reactions	

\* Pwo DNA Polymerase, Pwo SuperYield DNA Polymerase, Pwo Master, and Expand Reverse Transcriptase are not available in the U.S.A.



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## D. Abbreviations

<b>aa</b>	amino acid(s)
<b>ACP-T</b>	anchor ACPT, constant reverse primer
<b>ADP</b>	adenosine diphosphate
<b>AFLP</b>	Amplified fragment length polymorphism
<b>AMP</b>	adenosine monophosphate
<b>AP</b>	alkaline phosphatase
<b>AP-PCR</b>	arbitrarily primed PCR
<b>ARMS</b>	Amplification refractory mutation system
<b>ATP</b>	adenosine triphosphate
<b>ATPase</b>	adenosine triphosphatase
<b>β-Gal</b>	β-Galactosidase
<b>bp</b>	base pair
<b>BRCA1</b>	breast cancer susceptibility gene
<b>BrdU</b>	bromodeoxyuridine
<b>BSA</b>	bovine serum albumin
<b>°C</b>	degree Celsius
<b>CaCl<sub>2</sub></b>	Calcium chloride
<b>cAMP</b>	cyclic AMP
<b>cDNA</b>	complementary DNA
<b>conc.</b>	concentration
<b>CP</b>	crossing point
<b>CTP</b>	cytidine triphosphate
<b>D</b>	Dalton
<b>DAPI</b>	4'-6-diamidino-2-phenylindole
<b>dATP</b>	desoxy ATP
<b>DMSO</b>	dimethyl sulfoxide
<b>DNA</b>	deoxyribonucleic acid
<b>DNase</b>	deoxyribonuclease
<b>DOP – PCR</b>	Degenerate Oligonucleotid Primer – PCR
<b>DTE</b>	Dithioerythriol
<b>DTT</b>	dithiothreitol
<b>dU</b>	2'-deoxy uridine nucleoside
<b><i>E. coli</i></b>	Escherichia coli
<b>EDTA</b>	ethylenediaminetetraacetic acid
<b>EGTA</b>	ethyleneglycol-bis (beta-aminoethylether)- N,N'-tetraacetic acid
<b>ELISA</b>	enzyme-linked immunosorbent assay
<b>EM</b>	electron microscopy
<b>ER</b>	endoplasmic reticulum
<b>FACS<sup>®</sup></b>	BD fluorescence-activated cell sorter
<b>FBS</b>	fetal bovine serum
<b>FCS</b>	fetal calf serum
<b>FGF</b>	fibroblast growth factor
<b>FISH</b>	fluorescent in situ hybridization
<b>FITC</b>	fluorescein isothiocyanate
<b>g</b>	gram
<b><i>g</i></b>	unit of gravity
<b>GFP</b>	green fluorescent protein
<b>GMP</b>	Good Manufacturing Practice

<b>GST</b>	glutathione S-transferase
<b>GTP</b>	guanosine triphosphate
<b>h</b>	hour
<b>HA</b>	hemagglutinin
<b>Hepes</b>	N-2-hydroxyethylpiperazine-N'-2-ethane sulfonic acid
<b>His<sub>6</sub></b>	hexahistidine
<b>HPLC</b>	high performance liquid chromatography
<b>HRP</b>	horseradish peroxidase
<b>HTS</b>	High throughput screening
<b>HY</b>	High yield
<b>Hz</b>	Hertz (Frequency)
<b>IEF</b>	isoelectric focusing
<b>IFN</b>	interferon
<b>Ig</b>	immunoglobulin
<b>IMAC</b>	Immobilized metal affinity chromatography
<b>IPTG</b>	isopropyl-β-D-thiogalactoside
<b>kb</b>	kilobase(s)
<b>kbp</b>	kilobase pair(s)
<b>kD</b>	kilo Dalton(s)
<b>LCM</b>	laser capture microdissection
<b>LED</b>	light emitting diode
<b>μl</b>	microliter(s)
<b>ml</b>	milliliter(s)
<b>m</b>	meter
<b>μm</b>	micrometer(s)
<b>M</b>	molar
<b>MES</b>	2-(N-morpholino)ethane sulfonic acid
<b>min</b>	minute
<b>mol</b>	mole(s)
<b>MOPS</b>	4-Morpholino propane sulfonic acid
<b>mRNA</b>	messenger RNA
<b>MW</b>	molecular weight
<b>MP</b>	microplate
<b>N</b>	normal (concentration of ionizable groups)
<b>Ni-NTA</b>	Nickel-Nitrilotriacetic acid
<b>NMR</b>	nuclear magnetic resonance
<b>No.</b>	number
<b>NTP</b>	Nucleoside triphosphate
<b>OD</b>	optical density
<b>OEL</b>	overlapping extension ligation
<b>ORF</b>	open reading frame
<b>PAGE</b>	polyacrylamide gel electrophoresis
<b>PBS</b>	phosphate-buffered saline
<b>PCA</b>	perchloric acid
<b>PCR</b>	polymerase chain reaction
<b>PDGF</b>	platelet-derived growth factor
<b>pH</b>	hydrogen ion concentration, -log[H <sup>+</sup> ]
<b>PKC</b>	protein kinase C
<b>PMSF</b>	phenylmethylsulfonyl fluoride
<b>POD</b>	horseradish peroxidase

<b>PVDF</b>	polyvinylidene fluoride
<b>Pwo</b>	Pyrococcus woesei
<b>qPCR</b>	quantitative PCR
<b>RACE</b>	rapid amplification of cDNA ends
<b>RAS</b>	Roche Applied Science
<b>RBS</b>	ribosomal binding site
<b>RER</b>	rough endoplasmic reticulum
<b>RFLP</b>	Restriction fragment length polymorphism
<b>RIA</b>	radioimmunoassay
<b>RNA</b>	ribonucleic acid
<b>rPCR</b>	reverse PCR
<b>RNase</b>	ribonuclease
<b>ROX</b>	6-carboxy-X-rhodamine
<b>RT-PCR</b>	reverse transcriptase PCR
<b>RU</b>	response units
<b>s</b>	seconds(s)
<b>SA</b>	streptavidin
<b>SD</b>	Shine Dalgarno sequence
<b>SDS</b>	sodium dodecyl sulfate
<b>SSC</b>	standard saline citrate
<b>SSCP</b>	Single Strand Conformation Polymorphism
<b>ssDNA</b>	single stranded DNA
<b>T7</b>	Phage T7
<b>TAE</b>	Tris-acetate-EDTA
<b>Taq</b>	Thermus aquaticus
<b>TBE</b>	Tris-borate-EDTA
<b>TBS</b>	Tris-buffered saline
<b>TCA</b>	trichloroacetic acid
<b>TdR</b>	thymidine deoxyribose
<b>TE</b>	Tris-EDTA
<b>TGF</b>	transforming growth factor
<b>Tgo</b>	Thermococcus gorgonarius
<b>TLC</b>	thin layer chromatography
<b><math>T_m</math></b>	melting temperature
<b>TNF</b>	tumor necrosis factor
<b>tPA</b>	Tissue Plasminogen Activator
<b>Tris</b>	tris(hydroxymethyl)aminomethane
<b>tRNA</b>	transfer RNA
<b>Tth</b>	Thermus thermophilus
<b>TUNEL</b>	Tdt-mediated dUTP-biotin nick end labeling
<b>U</b>	unit
<b>UDP</b>	uridine diphosphate
<b>UNG</b>	Uracil-DNA N-glycosylase
<b>UTP</b>	uridine triphosphate
<b>UTR</b>	untranslated region
<b>UV</b>	ultraviolet
<b>V</b>	volt
<b>Vol.</b>	volume
<b>W</b>	watt
<b>Wt</b>	wild type

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## F. Index

<b>Index-Key</b>	<b>Page</b>
Absolute Quantification .....	204
Additives .....	35
Activation time.....	292
Activity of Restriction Enzymes .....	189
Affinity purification .....	48
Additives .....	122, 233, 240
Agarose gel.....	60, 72, 100, 159, 165, 172, 174
Agarose Gel DNA Extraction Kit .....	172
Amount of DNA Template .....	33
Amplification .....	9, 10, 30, 31
AMV Reverse Transcriptase.....	129, 137
Analysis.....	195, 196, 197, 207
anchor ACP-T .....	259
Annealing of primers .....	10
Bacterial DNA .....	46
Basic PCR .....	65
Bisulfite Sequencing.....	258
Blends .....	30
Blunt-end .....	90
Blunt-end cloning.....	175, 176
Blunt-end ligation.....	176
BRCA1 .....	258
Breast cancer susceptibility gene, BRCA1,.....	271
Carryover	
- contamination .....	108, 287
- prevention.....	26, 30, 34, 65, 108, 287
cDNA .....	9, 31, 33, 46, 60
Chemically modified.....	29
Cleaning/Decontaminating the laboratory environment.....	24
Cloning.....	78, 175, 177
C. therm. Polymerase .....	129
- One-Step RT-PCR System.....	129, 140



<b>Index-Key</b>	<b>Page</b>
Comparison of	
- PCR.....	32
- RT-PCR Enzymes .....	32
Competitive PCR .....	195
Competitive RT-PCR Assay.....	273
Concentration of Deoxynucleoside Triphosphate.....	34
Concentration of Magnesium .....	34
Constant reverse primer .....	259
Contamination .....	19, 20, 23, 53, 287, 291
Controls .....	25
Cosmids .....	180
CpG Island.....	258
Cross-contamination .....	19, 21, 23
Crossing point (CP) .....	203
Cycle conditions .....	286
Cycle number .....	33
Cycling Parameters.....	36, 36
Deoxyuridine triphosphate (dUTP) .....	108
Denaturation .....	10, 123
- temperature.....	123
Designing Primers.....	82
Difficult (GC-rich) DNA templates .....	65, 238, 287, 289
Dimers .....	286
Direct Colony PCR.....	247
Disposables .....	24, 28
Distribution of G/C and A/T rich domains.....	41
DNA isolation from an agarose gel slice .....	160
DNA .....	49
- Bacterial.....	46
- Isolation.....	47
- Methylation.....	250
- Template.....	288
- Plasmid.....	46
dNTP.....	34
End-point analysis.....	195
Enzyme concentration .....	290

<b>Index-Key</b>	<b>Page</b>
Exonuclease .....	9, 30, 86, 176, 218
Expand 20 kb <sup>PLUS</sup> PCR System .....	65, 92
Expand Cloning Kit .....	180
Expand High Fidelity PCR System.....	30, 65, 79, 86
Expand High Fidelity <sup>PLUS</sup> System.....	113
Expand Long Range, dNTPack .....	30, 65, 92, 94
Expand Reverse Transcriptase .....	129, 145, 151
Expand	
- Vector I.....	181
- Vector II .....	181
- Vector III .....	181
Extension .....	10
Extraction from Agarose Gel Slices.....	165
FastStart High Fidelity Enzyme .....	30
FastStart High Fidelity PCR System.....	65, 79, 83, 108
FastStart High Fidelity System .....	79, 230
FastStart PCR Master.....	66, 79, 119, 244
FastStart Taq DNA Polymerase .....	29, 43, 65, 66, 238, 242, 251, 261
Multiplex PCR optimization .....	230
Fluorescein .....	199, 202
Fluorescent Analysis of a PCR Cycle.....	196
FRET Principle.....	199
GC content .....	41, 110, 143, 154, 229, 236, 262
GC-RICH PCR System .....	30, 65
Gel filtration.....	49
GMP Grade .....	74
GeneFishing .....	259, 261
Genomic DNA .....	291
Genopure Plasmid Kit Maxi.....	163
Glass fiber fleece.....	160
Good Primer Design.....	41
Hexamer primers .....	292
High Fidelity Amplification .....	81
High Fidelity PCR.....	65
High Fidelity PCR Master .....	79, 119



<b>Index-Key</b>	<b>Page</b>
High Pure	
- High Pure 96 UF Cleanup Kit .....	159
- High Pure 96 UF Cleanup System .....	167
- High Pure PCR Product Purification Kit .....	159
- High Pure Viral Nucleic Acid Kit .....	50
High throughput purification .....	167
Homogeneous PCR .....	195
Hot Start .....	29, 65, 66, 97
Hot Start PCR .....	66
Inhibitors of RT reaction .....	290
Initial multiplex PCR optimization .....	230
Isolation of	
- cDNA .....	60
- DNA .....	172
- Nucleic Acids .....	54
Laboratory clothing .....	24
Laboratory facilities .....	19, 20
Lambda packaging system .....	180
Laser capture microdissection .....	242
License Disclaimer .....	335
LightCycler®	
- 480 System .....	213
- 480 SYBR Green I Master .....	221
- Carouel-based System .....	211
- FastStart DNA Master .....	215, 216
- Genotyping Master .....	221
- Instruments .....	211
- Multiplex Master HybProbe .....	218
- Probes Master .....	221
- TaqMan® Master .....	217
Long template .....	30, 65
Long template PCR .....	65
Low melting point agarose .....	159
M-MuLV Reverse Transcriptase .....	129
MagNALyser Instrument .....	51, 53
MagNA Pure Compact .....	51, 53
MagNA Pure LC .....	51, 52
Magnesium .....	33, 34
Manual preparation .....	48
Master Mixes for One-step RT-PCR .....	219

<b>Index-Key</b>	<b>Page</b>
Master Mixes for Two-Step RT-PCR.....	220
Medium- and High-Throughput Applications.....	213
Melting Curve Analysis.....	207
Melting temperature.....	42
Methylated CpGs.....	254
Methylation-specific PCR.....	250, 251
Mineral oil.....	28
Mixtures.....	30
MgCl <sub>2</sub> concentration.....	286, 288, 289, 291
Modified nucleotides.....	129
Monoplex PCR.....	230
M-PCR.....	250
Multiplex PCR.....	30, 65, 230
Nuclease.....	45
Nucleic acids.....	52, 54
Nucleotides.....	33
Nonspecific binding.....	287, 292
One-Step	
- Procedure.....	128
- RT-PCR.....	130
Optimal	
- Annealing temperature.....	36
- Elongation time.....	36
- Number of cycles.....	36
Optimization.....	120, 123
Optimization of Multiplex PCR.....	232, 233
Optimizing the Mg <sup>2+</sup> Concentration.....	120
Ordering Information.....	299
pCAP <sup>s</sup> .....	178, 179, 246
PCR Cloning Kit (Blunt End).....	178
PCR Master	
- Applications.....	14
- Based Methylation Assay.....	254
- Cycle.....	9
- Enzymes.....	11
- Equipment.....	13
- Laboratory.....	19, 20, 21
- Mix.....	189
- Templates.....	45

<b>Index-Key</b>	<b>Page</b>
PCR Core Kit.....	65, 108, 110
PCR Protocol Selection Guide.....	65
PCR Reagents for the LightCycler® 480 System .....	221
PCR set-up.....	25
Plasmid DNA.....	46
Plasticware .....	28
Polyadenylation (pA) sites .....	265
Positive controls .....	25, 137
Positive Selection Vectors.....	177, 178
Presence of additives .....	240
Preventing Carryover .....	65, 108, 218
Primer .....	286
- annealing.....	10, 36, 44
- concentration .....	44, 286, 291
- design .....	41, 42, 286, 291
- dimer .....	41, 291
- extension .....	44
- problems.....	288
- quality.....	286
- specificity .....	286
Primers for Reverse Transcription.....	130
Processing of PCR Products .....	72
Protector RNase Inhibitor .....	134
Product Analysis.....	28
Proofreading.....	29, 30
Properties of Reverse Transcriptases .....	129
Protocols .....	65, 130
Purification and Cloning of PCR Product.....	246
Purification from Restriction Nuclease Digest Mix .....	164
Purification of Large DNA Fragments .....	163
Purity of product.....	167
Pwo DNA Polymerase	
- Pwo SuperYield DNA Polymerase .....	29, 65, 79, 81, 190
- Pwo SuperYield DNA Polymerase, dNTPack.....	29
Qualitative Real-Time Analysis .....	195
Quantitative analysis .....	195

<b>Index-Key</b>	<b>Page</b>
Quick Spin.....	49
Rapid DNA Ligation Kit.....	182
Reaction temperature .....	290
Real-Time PCR .....	37
Recovery of PCR .....	159
Recovery rate.....	169
References.....	318
Relative Quantification.....	206
Repetitive sequences .....	102
Restriction digest fragments.....	172
Reverse transcription of total RNA into cDNA.....	267
Retrovirus vector system .....	265
RNA .....	49
- Isolation .....	47
- template .....	133, 290
RNase.....	129, 290
RT-PCR .....	9, 32, 128, 130, 263, 267
Sample containers.....	28
Sample materials .....	51
Secondary amplification product .....	287
Secondary structure .....	41, 290
Separation of Work Areas.....	20
Sequence-Independent Detection Assays .....	196
Sequence-Specific Probe Binding Assays.....	199
Silica matrix .....	172
Size exclusion .....	167
Sizes of amplicons .....	292
Silica adsorption .....	48
Small amount of DNA.....	233
Software programs.....	41
Solution-based isolation .....	48
Specificity .....	242
Splicing events.....	266



<b>Index-Key</b>	<b>Page</b>
Storage .....	89
- conditions .....	285
Supplement.....	122
SYBR Green I.....	198
T4 DNA Ligase .....	182
T4 DNA Polymerase.....	178, 246
TA Cloning .....	78, 175
Taq DNA Polymerase .....	29, 43, 65, 74
- GMP Grade .....	74
Template	
- amount .....	46
- preparation.....	49
- RNA .....	133
- quality.....	285
Template Dilution Buffer .....	67
Terminal transferase activity.....	175
Thermal Cyclers .....	27, 28
Thermostable.....	29, 86, 113, 140, 147, 219
Tth DNA Polymerase .....	129
Titan One Tube Kit or System .....	135
Titan One Tube RT-PCR.....	135, 140
Total RNA isolation .....	262
Transcriptor First Strand cDNA Synthesis Kit.....	145, 147, 148
Transcriptor Reverse Transcriptase .....	129, 145, 149, 262
Troubleshooting .....	71, 77, 89, 99, 106, 115, 285
Two-Step Procedure .....	128
- RT-PCR .....	130
Ultrafiltration .....	167
Universal Probe Library .....	202
Uracil-DNA-Glycosylase-mediated cloning .....	176
Uracil-DNA N-glycosylase (UNG).....	108
Water.....	24
Work Guidelines.....	22

## Index of authors

Anton, Markus	Roche Applied Science, Germany	276-281
Bekele, Yalem, Z.,	Wellcome Trust Sanger Institute, Hinxton, Cambs, UK	276-281
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Dante, Robert	Université Centre Léon Bérard, Lyon, France	254-258, 271-275
Deniz, Sibel	Roche Applied Science, Germany	244-249
Foernzler, Dorothee	Roche Diagnostics, Switzerland	238-241
Franke, Isabel	Roche Applied Science, Germany	244-249
Green, Andy	University of Leicester, UK	242-243
Harrison, Elliot, S., I.	Wellcome Trust Sanger Institute, Hinxton, Cambs, UK	276-281
Hermann Danielle	Roche Diagnostics, Switzerland	238-241
Hloch, Brigitte	Roche Applied Science, Germany	230-237
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Sobek, Harald	Roche Applied Science, Germany	175-177, 244-249
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12. Under license from Gilead Sciences Inc.

**Notice to the purchaser:**

Pwo DNA Polymerase, Pwo SuperYield DNA Polymerase, Pwo Master, and Expand Reverse Transcriptase are not available in the U.S.A.









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