

Simple enzymatic means to neutralize DNA contamination in nucleic acid amplification

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Reverse transcription PCR (RT-PCR) is prone to false positives when contaminating DNA molecules are present at the start of a reaction. Contaminants that derive from earlier work using a given primer pair (carryover PCR products) are of particular concern when those primers are used routinely, as in clinical diagnostics or environmental monitoring. In addition, contamination by genomic DNA can significantly interfere with quantitative and qualitative analysis of RNAs by RT-PCR. Here we describe contaminant restriction (ConR), a method that can be used to neutralize carryover and genomic DNA contamination in RT-PCR studies. Restriction enzymes (REs) added to the amplification cocktail cleave contaminant DNA molecules while sparing the intended target nucleic acid. Restriction, reverse transcription, and amplification steps all take place in the same sealed vessel, thus avoiding any danger of recontamination. ConR eliminates carryover contamination in PCR without compromising target sequence amplification. Because the method is effective against both genomic and carryover contamination, it can be employed routinely in one-step RT-PCR, whatever the RNA target or the nature of the potential DNA contaminant. A variation of this decontamination method, amplicon primer site restriction (APSR), is effective specifically against carryover contamination. APSR, unlike ConR, can be applied during PCR-based amplification of DNA target molecules.

INTRODUCTION

Reverse transcription-coupled PCR (RT-PCR) allows vanishingly small amounts of target RNAs to be amplified indefinitely. However, this sensitivity also allows trace contamination from earlier PCR experiments (carryover PCR products) to be amplified instead of, or in addition to, the desired target. Such carryover contamination is a frequent source of false-positive results in clinical analysis (1). Genomic DNA contamination can pose a related difficulty (2), especially when primers lie within a single exon, rendering RNA and genomic DNA PCR products indistinguishable. Although various practices can control carryover and genomic DNA contamination, these problems persist in many laboratories.

Here we describe an enzymatic method we developed to eliminate contaminant amplification when amplifying cellular or viral RNAs by RT-PCR. This procedure, contaminant restriction (ConR), uses restriction enzymes (REs) to cleave double-

stranded DNA (dsDNA) contaminants while sparing the intended RNA target and the primer DNA molecules. ConR employs a cocktail of frequently cutting REs, which are added to the standard components of a single-tube, one-step RT-PCR mixture. Together, these enzymes act as a highly promiscuous nuclease that degrades dsDNA contaminants associated with template nucleic acids, PCR primers, or other reaction components while leaving the RNA template and the amplification primers intact. ConR is carried out in the same sealed vessel where thermal cycling takes place, thus avoiding any opportunity for recontamination to occur during the reaction.

While ConR can be safely included in all RT-PCR applications, it is incompatible with PCR, where target DNA molecules would be neutralized by the RE cocktail along with other contaminating dsDNAs. However, a related method, termed amplicon primer-site restriction (APSR), can be used to control carryover contamination in advance of DNA

amplification. The APSR technique, described in the supplementary material to this report (available online at www.BioTechniques.com), requires PCR primers to be engineered to carry at their 5' ends recognition sites for a Type IIS RE. Because these REs, by definition, cleave at a site distinct from the recognition sequence, preincubation of PCR mixtures with the corresponding Type IIS enzyme creates double-stranded breaks in previously generated PCR products, thus removing some or all of the sequence to which primers would otherwise anneal. APSR therefore neutralizes any carryover PCR products that may be present before thermal cycling is initiated, but it does not interfere with amplification of the desired DNA templates.

MATERIALS AND METHODS

Restriction Enzymes

AluI, *NlaIII*, *Sau96I*, and *StyD4I* (New England Biolabs, Beverly, MA, USA) were mixed to produce a stock RE cocktail containing 2 U/ μ L for each enzyme, or 8 total RE U/ μ L. Except where noted, the RE cocktail was used as a 40 \times stock (i.e., 4 total RE U/20 μ L reaction).

RNA Targets and DNA Contaminants

Model "contaminant" and "target" sequences were generated in vitro from the pBluescribe-based plasmids pBS(5'ac) (3) and pBS(MLC1v) (4), which were gifts of Ingo von Both (Samuel Lunenfeld Research Institute, Toronto, ON, Canada). Contaminant H was generated using the pBluescribe polylinker-directed primers PLHga_Sense (5'-AAAAAGACGCGGGAA-CAAAAGCTTGCATGC-3') and PLHga_Antisense (5'-AAAAAGAC-GCGAATTCGAGCTCGGTACCC-3') to amplify a 231-bp fragment containing the pBS(5'ac) insert, which was gel purified and quantified by spectrophotometry. To produce a model RNA template that could be amplified by RT-PCR using the PLHga primer pair, a 657-bp *PvuII* fragment containing the insert region of pBS(MLC1v) was

transcribed in vitro using T7 RNA polymerase (Roche Applied Science, Indianapolis, IN, USA). The resulting 544 nucleotide transcript was treated with DNase I, purified over an RNeasy® column (Qiagen, Valencia, CA, USA), and quantified. Tissue culture-derived total RNA and genomic DNA were prepared from confluent MCF7 cells using TRIzol™ extraction (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions.

ConR Treatment and RT-PCR Amplification

Except where noted, target and contaminant sequences were amplified on an MJ Research PTC-025 DNA Engine Tetrad™ thermal cycler (Bio-Rad Laboratories, Hercules, CA, USA), using the One-Step RT-PCR kit (Qiagen), as follows. Standard 20-μL reactions were carried out according to the manufacturer's recommendations, except that reaction mixtures were supplemented with 4 U RNase Inhibitor (MBI Fermentas, Hanover, MD, USA) and the indicated concentration of restriction enzymes. Amplification mixtures, containing target and/or contaminant sequences, as well as primers, enzymes, and other standard reaction components, were incubated at 37°C for 1 h (restriction), followed by 30 min at 50°C (reverse transcription), 10 min at 94°C (inactivation of REs/activation of the DNA polymerase), and 45 cycles of 3-step thermal cycling (94°C for 30 s, 60°C for 30 s, and 68°C for 30 s). RE storage buffer [50 mM KCl, 10 mM Tris-HCl, pH 7.4, 0.1 mM EDTA, 1 mM dithiothreitol (DTT), and 50% glycerol] was used as an enzyme diluent when necessary and served as a negative control in samples not treated with REs. For competitive RT-PCR experiments, in vitro transcribed RNA template and Contaminant H were amplified with the PLHga primer pair, producing a 361-bp RT-PCR product and a 231-bp contaminant band, respectively.

For studies of genomic DNA decontamination, MCF7 RNA and DNA were amplified using the primers CypF (5'-TTCCGACACTCTTCCTTCGT-

3') and CypR2 (5'-GCTCACAGCAGGCATGCTTCA-3'), which hybridize to different exons in the human cytochrome P450 gene *CYP1A1* (5). These same *CYP1A1*-specific primers and MCF7 RNA were also used in real-time RT-PCR. In these experiments, a probe specific for genomic DNA (TaqmanG; 5'-DFAM-GAGATTG-CCTGTTGCCCTGAGCCTG-DTAM-3') or for the spliced messenger RNA (mRNA) (TaqmanC; 5'-DFAM-CATCCCCCACAGCACAA-CAAGAGACAC-DTAM-3') were added to the standard TaqMan® One-Step Real-Time RT-PCR kit (Applied Biosystems, Foster City, CA, USA) reaction mixture, supplemented with the RE cocktail or RE storage buffer as indicated. Following a 1-h ConR step, reactions proceeded according to the manufacturer's recommendations, using 45 cycles of 3-step thermal cycling as above.

RESULTS AND DISCUSSION

The RE mixture developed for ConR contains *AluI*, *NlaIII*, *Sau96I*, and *SlyD4I*. These enzymes were selected because they are frequent cutters and cut efficiently at 37°C in reaction buffers from various commercial RT-PCR systems (data not shown).

Because they are fully inactivated at higher temperatures, they do not act on dsDNAs produced following reverse transcription and thermal cycling.

To test whether ConR using these four REs could control carryover contamination, we developed a model target RNA (RT-PCR product size: 361 bp) and a shorter dsDNA fragment (Contaminant H: 231 bp) that can be amplified by the same primer pair. Without ConR pretreatment, the presence of 24,000 copies of Contaminant H almost completely prevented amplification from the target RNA (Figure 1). However, ConR eliminated contaminant amplification under these conditions, permitting efficient amplification from the RNA target. With severe contamination (600,000 copies of Contaminant H; molar ratio of target to contaminant, 100:1), target amplification was fully blocked in the absence of ConR treatment. Although ConR did not entirely eliminate dsDNA contaminant amplification in the latter case, the process still greatly enhanced amplification from the RNA template. Conversely, where bona fide carryover occurred—as distinct from the deliberate introduction of a competing target molecule—ConR appeared to neutralize the contamination completely. Thus, in the experiment shown, the water blank yielded a product that co-migrated

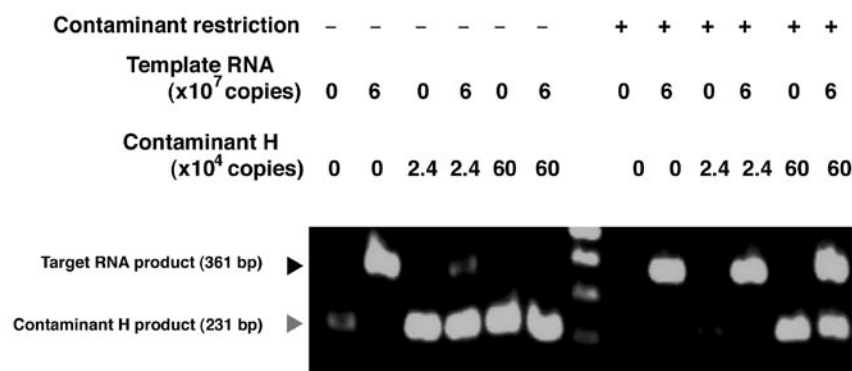


Figure 1. RT-PCR analysis of carryover contamination following contaminant restriction (ConR). *AluI*, *NlaIII*, *Sau96I*, and *SlyD4I* were added to one-step RT-PCR mixtures containing an in vitro transcribed model target RNA (20 pg, or approximately 7×10^7 copies per reaction) and a model dsDNA (Contaminant H: 231 bp). Following a ConR preincubation step (1 h at 37°C), RT-PCR was performed. Note that the negative control sample (untreated water blank; leftmost lane) yielded a 231-bp product, suggesting that it contained trace amounts of Contaminant H as a result of bona fide PCR carryover. RT-PCR, reverse transcription PCR; dsDNA, double-stranded DNA.

with the expected Contaminant H PCR product, presumably a result of carryover from earlier experiments. In other applications of ConR (data not shown) or APSR (see the Supplementary Material), RE pretreatment has been similarly successful in abolishing contaminant amplification in water blanks.

Having established that ConR is effective against carryover contamination in RT-PCR, we considered whether genomic DNA contamination, which often complicates gene expression studies, could be neutralized in a similar manner. A total RNA preparation from MCF7 cells was used for the amplification of a fragment from the human CYP1A1 transcript. The RNA

was prepared using TRIzol, which can produce RNA significantly contaminated with genomic DNA (6) and was not DNase I treated. RT-PCR amplification using the primer set chosen yielded the two predominant products of the expected sizes: a processed mRNA PCR product (387 bp; Figure 2, black arrowheads) and a genomic DNA PCR product (725 bp; gray arrowheads). Amplification of pure human genomic DNA yielded only the latter (Figure 2B). Preincubation with the ConR RE cocktail abrogated genomic DNA amplification, both from DNA-contaminated total RNA (Figure 2A) and pure human genomic DNA (Figure 2B). The effect was seen over a wide range of input RE levels, from 1 to 10

U total RE activity per reaction. Only at the highest level tested (20 U/20 μ L reaction) did ConR inhibit RNA amplification (Figure 2A).

To test ConR's limits in controlling genomic DNA contamination, purified DNA was added to the MCF7 RNA preparation previously described. ConR allowed specific RNA amplification and greatly diminished genomic DNA amplification even in the presence of 100 pg additional genomic DNA in the 20- μ L amplification reaction (Figure 2C).

We also explored the possibility of replacing the ConR RE cocktail with a single, highly promiscuous RE, *Cvi*JI, which cleaves dsDNA on average every 64 bp. However, whereas the RE cocktail was effective against DNA contamination over a wide range of enzyme concentrations and interfered with RT-PCR only at extreme concentrations (Figure 2A), the range at which *Cvi*JI is beneficial appeared narrow (data not shown). Whether the observed interference with RNA amplification is an intrinsic property of *Cvi*JI or is due to a contaminating activity, such as an RNase, has not been resolved.

To evaluate whether ConR compromises the efficiency of RNA amplification (e.g., by degrading input RNA template or first-strand cDNA molecules), we included a ConR step in real-time RT-PCR analysis. To this end, we developed TaqMan probes that could distinguish CYP1A1 PCR products generated from genomic DNA or from the spliced transcript. MCF7 genomic DNA and genomic DNA-contaminated RNA were amplified using the CYP1A1-specific primers described above, and products were detected using these probes. As shown in Figure 3A, ConR effectively blocked the amplification of either pure genomic DNA or genomic DNA contaminants in this system. Conversely, ConR enhanced amplification from the CYP1A1 mRNA (Figure 3B). Taken together with the previous results, this finding suggests that possible undesirable side effects of ConR treatment are negligible relative to the benefit of neutralizing admixed DNA molecules, which could otherwise consume dNTPs or PCR primers during amplification.

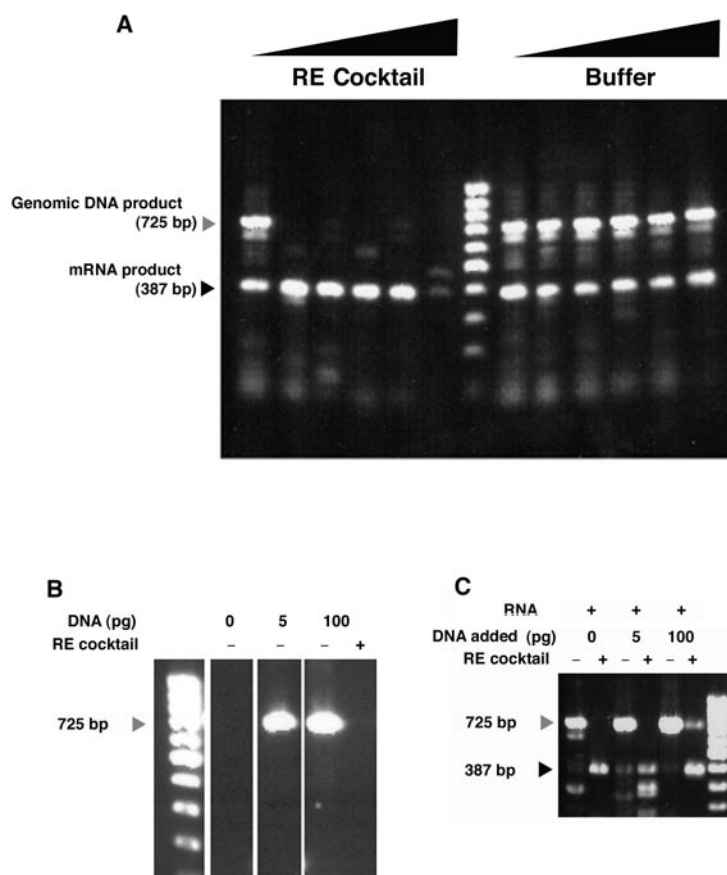


Figure 2. Control of genomic DNA by ConR. (A) RNA samples (≤ 4.4 ng total RNA, admixed with genomic DNA) were incubated with 0, 1, 2.5, 5, 10, or 20 U of total RE activity per reaction prior to reverse transcription and PCR (45 cycles). Control reactions contained equivalent volumes of RE storage buffer. (B) Amplification of similarly treated samples of pure genomic DNA in the presence (+) of RE cocktail (2.5 U total RE/20 μ L RT-PCR). (C) Amplification of similarly treated RNA samples (≤ 6 ng total RNA, admixed with genomic DNA), supplemented with the indicated amounts of pure genomic DNA in the presence (+) of RE cocktail (2.5 U total RE/20 μ L RT-PCR) or RE storage buffer (-). ConR; Contaminant restriction; RE, restriction enzyme; RT-PCR, reverse transcription PCR; mRNA, messenger RNA.

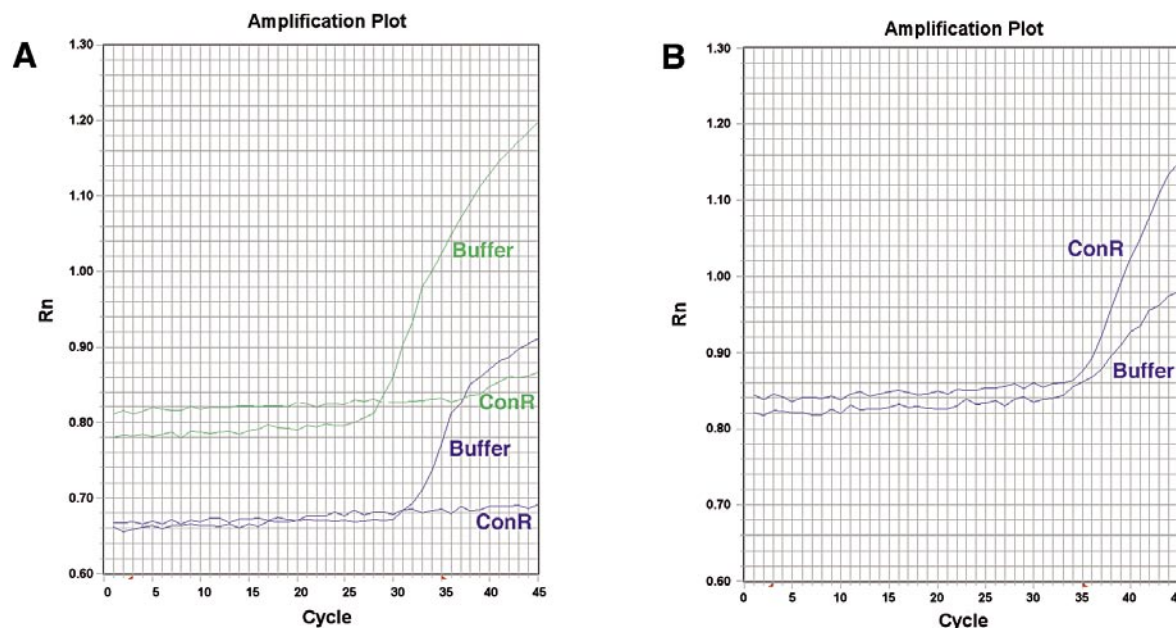


Figure 3. Real-time RT-PCR detection of genomic DNA and mRNA amplification following ConR. (A) Detection of a genomic DNA PCR product from the *CYP1A1* gene, using the TaqmanG probe. Green traces: amplification of human genomic DNA, corresponding to 100 genome equivalents per sample, in the absence (Buffer) or presence (ConR) of RE cocktail. Blue traces: amplification of human genomic DNA contaminant in MCF7 total RNA (≤ 4.4 ng total RNA, admixed with genomic DNA). (B) Detection of a spliced PCR product from the *CYP1A1* mRNA, using the TaqmanC probe. MCF7 total RNA (≤ 4.4 ng total RNA, admixed with genomic DNA) was amplified in the absence (Buffer) or presence (ConR) of RE cocktail. RT-PCR, reverse transcription PCR; mRNA, messenger RNA; ConR, contaminant restriction; RE, restriction enzyme.

As an early review on the subject (7) recognized, an ideal solution to the problem of carryover contamination would couple decontamination and amplification so that reaction mixtures freed of carryover PCR products cannot be recontaminated before the end of the procedure. The widely used dUTP/uracil-DNA glycosylase (UDG) technique (8,9) is one example of such an approach. The UDG method, which can be applied to one-step RT-PCR, employs a closed-tube format, where reagents are added only once, and any carryover PCR products are destroyed before amplification begins. However, inclusion of dUTP and/or UDG is incompatible with several commonly used PCR or RT-PCR procedures, particularly those that employ archaeal DNA polymerases (10). Moreover, for the UDG method to be effective, dUTP must be included each time a given primer pair is used. ConR (and likewise APSR; see the Supplementary Material) is also carried out in a closed-tube format, but, unlike the UDG method, can be safely omitted from routine amplification procedures

and applied whenever contamination becomes a concern.

RE-pretreatment to control DNA contamination in PCR reagents (11,12) is well suited to RT-PCR, as Dougherty et al. (13) first pointed out, because REs added immediately before amplification will destroy carryover contaminants without affecting first-strand cDNA or PCR primers. When the enzymes are introduced in a one-step RT-PCR procedure, as we describe, ConR pretreatment neutralizes genomic DNA along with any carryover contaminants present prior to reverse transcription (14).

Dougherty et al. (13) established that the addition of a restriction step to an RT-PCR protocol can eliminate carryover when even a single restriction site is present in the corresponding contaminant. However, to our knowledge, no one has previously developed REs as general-purpose reagents to be used in RT-PCR. In silico analysis of murine expressed sequence tags (ESTs; data not shown) suggests that our RE cocktail would neutralize 99.9% of all possible dsDNA contami-

nants ≥ 0.5 kb (a figure that could vary with GC content in other genomes). In our work with the severe acute respiratory syndrome (SARS) coronavirus (CoV; Chan et al., manuscript in preparation; Farcas et al., manuscript in preparation), we regularly amplify the 29,751 nucleotides of genomic RNA in overlapping approximately 0.5 kb PCR products and include ConR in each of 68 reactions. By monitoring water blank controls, we have found that this treatment successfully suppresses any carryover contamination.

Based on its ease of use and general applicability, we propose that ConR be applied routinely in one-step RT-PCR, whatever the RNA target or the nature of the potential DNA contaminant. However, because it employs promiscuous DNases, ConR is not appropriate for controlling carryover contamination in DNA amplification. APSR, which shares some of the advantages of ConR, offers a suitable alternative for carryover decontamination in PCR experiments.

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COMPETING INTERESTS STATEMENT

The authors declare no competing interests.

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