

# GeneSystem320™

Gene Expression Profiling System

## Instruction Manual

RAGEtag Synthesis Kit

801-0001, 801-1001-A

Human GS320 Standardization Kits

801-0004, 801-0005

Mouse GS320 Standardization Kits

801-0002, 801-0003

Rat GS320 Standardization Kits

801-0006, 801-0007

RAGE Primers

800-1005 thru 800-1332

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## Notices to Customer

Purchase of this kit does not constitute a license for PCR. A licensed polymerase and thermal cycler must be used in conjunction with this product. PCR is covered by patents owned by Hoffman-La Roche, Inc. and F. Hoffman-La Roche, Inc.

GS320 is licensed exclusively to KPL/Capital Genomix from MD Anderson Cancer Center. The RAGE technology is covered under US patent number 62216000 and one or more patents pending. Purchase of this product conveys a limited license for use of this product by the purchaser for basic research. Purchasers wishing to use this product for commercial research and/or development should contact Capital Genomix for a commercial license.

The product listed herein is for research use only and is not intended for use in human or clinical diagnosis. Nothing disclosed herein is to be construed as a recommendation to use this product in violation of any patents. The information presented above is believed to be accurate. However said information and product are offered without warranty or guarantee since the ultimate conditions of use and the variability of the materials treated are beyond our control. We cannot be responsible for patent infringements or other violations that may occur with the use of this product. No claims beyond replacement of unacceptable material or refund of purchase price shall be allowed.

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# 1 INTRODUCTION

## 1.1 Overview of Technology

The GeneSystem320™ (GS320) gene expression analysis system is based upon the technology developed at the MD Anderson Cancer Center (University of Texas) by Dr. Michael MacLeod. This system, also known as RAGE (rapid analysis of gene expression) and combinatorial oligo PCR, enables the analysis of virtually any gene or set of genes using a defined set of reagents including 320 PCR™ primers. It follows a simple protocol that yields useful data on mRNA expression profiles within a couple of weeks in the lab.

The object of GS320 is to provide a method for gene expression analysis that exceeds the capabilities of the state of the art. The GS320 technology is rapid and cost-effective, allows for easily reproducible results, has an adequate sensitivity to detect and quantify moderately rare transcripts, and identifies amplification products without additional cloning or sequencing steps. GS320 is flexible enough to analyze either a subset or virtually the complete genome.

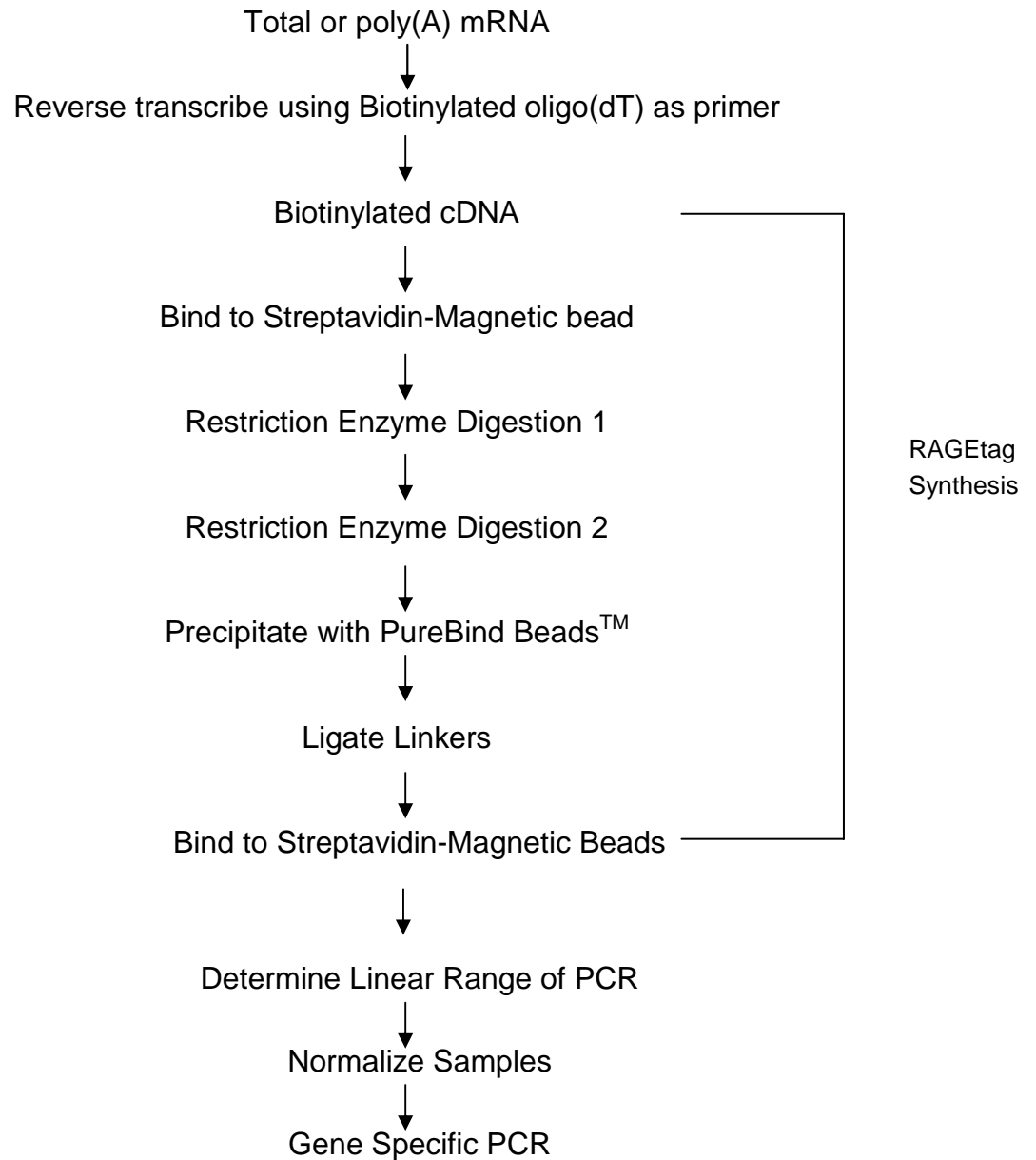
GS320 has the capability for detecting the frequency distribution of all polyadenylated mRNAs in a sample at any selected time. The method reduces the complexity of analysis because only a single unique fragment is derived from each molecular species of polyadenylated mRNA. The genome or a subset can be analyzed with a single set of reagents and reaction conditions. The technique allows for multiple samples to be analyzed simultaneously. The results generated from GS320 are proportional to the level of expression of the particular gene.

GS320 utilizes a defined set of 320 PCR primers and a unique message fragmentation protocol that enables analysis of virtually any eukaryotic gene or gene set. The protocol involves isolation of actively transcribing genes and recovery of a single fragment per mRNA molecule, conducting specific combinatorial oligo PCR on the fragments and then positively identifying the genes (PCR products) using dedicated software. The unique gene-specific fragments, called RAGEtags, are generated from mRNA using reverse transcription, a pair of restriction enzymes and 2 universal linkers.

RAGEtags may be generated in two orientations, A/B and B/A. The orientation refers to the relative location of the 3' most paired *Hsp92II* and *DpnII* sites to each other and the poly(A) tail. The fragments of cDNA isolated by digestion with *Hsp92II* and ligation to the A linker followed by digestion with *DpnII* and ligation to the B linkers are referred to as A/B RAGEtags. The second orientation of RAGEtags addresses cDNA species that have the *Hsp92II* recognition site in proximity to the poly(A) tail. B/A RAGEtags are obtained by reversing the order of restriction digestion and linker ligation. The linkers are composed of common PCR primer annealing sites and four base overhangs complementary to the ends created by restriction with either *Hsp92II* (A-linker) or *DpnII* (B-linker).

The GeneSystem320™ Database Search Engine was developed to provide mRNA sequence data specific to GS320. The program can be used in two ways: to predict the pair of RAGE primers and the RAGEtag orientation to be used for amplification of each specific gene, and to determine the identity of RAGE amplimers after combinatorial RAGE analysis. The program utilizes sequence data collected from the NCBI Entrez GenBank and UniGene databases which is processed to extract the data appropriate to GS320. The data depends on the integrity of the 3' end of the mRNA and includes a validation system to assess sequences for the likelihood that the last nucleotides in the sequence do in fact represent the 3' end. A verification process is employed to select a representative sequence from each UniGene cluster that is most likely to correspond to a mature mRNA including the 3' end. The database includes data for human, mouse and rat mRNAs. The database is updated monthly to add new and revised GenBank sequences and to maintain current UniGene data.

## 1.2 GS320 Protocol Flowchart



## 1.3 GS320 Protocol Overview

### A. Identify RAGE Primers to Amplify Specific Genes

The GS320 Database Analysis Software at [www.capitalgenomix.com](http://www.capitalgenomix.com) or [www.kpl.com](http://www.kpl.com) is used to identify the primers for amplifying genes. Refer to the GS320 Database Analysis Software Manual for directions. Note the orientation (A/B or B/A) of the RAGEtag preparation that is required for analysis of each specific gene.

### B. Synthesize cDNA containing a biotinylated oligo(dT). (Sections 3.1.1 and 6.2)

Either total or poly(A) mRNA is isolated by standard methods. Between 10-20 ug of total RNA or 2-5 ug of poly(A) mRNA is optimal for cDNA synthesis. cDNA is synthesized from the mRNA using a biotinylated oligo(dT) primer, which tags each cDNA molecule at the 3' poly(A) end.

### C. Prepare RAGEtags in the appropriate orientation. (Section 3)

Biotinylated cDNA is bound to a streptavidin magnetic bead. 1–2 ug of cDNA is used for RAGEtag synthesis.

For generation of A/B orientation RAGEtags, the immobilized cDNA is then cleaved with the restriction endonuclease *Hsp92II*, leaving a 4-base overhang upon digestion. All *Hsp92II* fragments except the 3' terminal fragment are released from the magnetic bead and discarded. The immobilized library of fragments are digested with a second 4-base overhang creating restriction enzyme, *DpnII*, releasing the fragments from the anchor. The released cDNA is precipitated using PureBind Beads™. A biotinylated A linker and unmodified B linker are then ligated to the digested ends of the released fragments. The ligated fragments are recovered using streptavidin magnetic beads.

For generation of B/A orientation RAGEtags, the immobilized cDNA is then cleaved with the restriction endonuclease *DpnII*, leaving a 4-base overhang upon digestion. All *DpnII* fragments except the 3' terminal fragment are released from the magnetic bead and discarded. The immobilized fragments containing *DpnII* ends are digested with a second 4-base overhang creating restriction enzyme, *Hsp92II*, releasing the fragments from the anchor. A biotinylated B linker and unmodified A linker are then ligated to the digested ends of the released fragments. The ligated fragments are recovered using streptavidin magnetic beads.

### D. Determine the linear range of PCR for RAGEtag preparation. (Section 4.1)

In order for GS320 to be considered semi-quantitative, it is necessary to ensure that the PCR reactions fall into the linear range. The GS320 Standardization Kits contain a panel of primer sets that amplify ribosomal protein genes and other housekeeping genes. These genes are used to determine the amount of RAGEtag used as template for PCR in order to maintain linear PCR product synthesis.

### E. Normalize the samples to one another using the standardization genes. (Section 4.2)

GS320 utilizes multiple external control genes for normalization of one sample to another. The GS320 Standardization Kits contain a panel of ribosomal protein genes and other housekeeping genes for quantitation of one sample to another. Using this panel, it is possible to find at least 4-5 genes that are constitutively expressed in the tissue or cells of interest. These genes can then be used to equalize one sample to another before performing differential GS320 analysis.

**F. Gene specific PCR using the appropriate RAGE primers.** (Section 5)

A combinatorial library of 320 primers is used to selectively amplify any genes of choice using the RAGEtag as the template. The PCR primers for GS320 are designed to provide specificity and uniform conditions in the PCR reactions. There are two sets of primers. Each set contains a common region derived from the linker. The 3' end of each primer comprises a specificity region of 3 to 4 nucleotides. The specificity region facilitates the amplification of a small number of different genes in a sample. There are a total of 256 A primers with 4-base specificity and 64 B primers with 3-base specificity. These primers may be combined pairwise with the two orientations of RAGEtag to produce  $(256 \times 64 \times 2 =)$  32,768 unique reactions. The presence of a relatively long common region (16 basepairs) in the RAGE primers allows optimal amplification with all primers under a single set of PCR conditions. For cDNAs in the Genbank database the pair of primers that will amplify the gene-specific target can be predicted from the sequence, as well as the size of the resulting amplicon. The intensity of the amplified product band on a polyacrylamide gel is the relative measure of the frequency of the corresponding mRNA in the total population of mRNAs.

## 2 MATERIALS AND EQUIPMENT

### 2.1 RAGEtag Synthesis Kit

#### **Product Code: 801-0001, 801-0001-A**

<b>Kit Component</b>	<b>Product Code</b>	<b>Volume</b>	<b>Temp</b>
Biotinylated Oligo(dT)	800-0001	35 uL	-20°C
2X Binding Buffer	800-0002	3 X 1.5 mL	2–8°C
Linker A	800-0003	25 uL	-20°C
Biotinylated Linker A	800-0074	25 uL	-20°C
Linker B	800-0004	25 uL	-20°C
Biotinylated Linker B	800-0075	25 uL	-20°C
SA-Magnetic Beads	800-0005	1 mL	2-8°C
<i>Hsp92II</i> Enzyme	800-0006	25 uL	-20°C
10X <i>Hsp92II</i> Buffer	800-0007	350 uL	-20°C
100X BSA	800-0008	13 uL	-20°C
<i>DpnII</i> Enzyme	800-0009	25 uL	-20°C
10X <i>DpnII</i> Buffer	800-0010	350 uL	-20°C
T4 DNA Ligase	800-0011	13 uL	-20°C
10X T4 DNA Ligase Buffer	800-0012	25 uL	-20°C
Glycogen	800-0014	45 uL	-20°C
5X PCR Buffer	800-0013	1.7 mL	-20°C
PureBind Beads™	800-0076	700 uL	2-8 °C

- Sufficient reagents are provided to prepare 5 RAGEtags.
- Reagents must be stored at the temperatures shown above.
- **Do not freeze the SA-Magnetic beads or PureBind Beads.**
- Store the –20°C reagents in a non-cycling freezer (not frost-free).
- The kit is stable for a minimum of 1 year from date of receipt when stored properly.
- Components can be purchased separately. Contact KPL technical services at 1-800-638-3167 for information pertaining to availability of individual kit components.

## 2.2 GS320 Standardization Kits

The GS320 Standardization Kits contain primer sets that amplify a variety of housekeeping genes and ribosomal protein genes. Using this panel of genes it is possible to find at least 4-5 genes that are constitutively expressed in the tissue or cells of interest. Amplification of these genes is used for determination of the quantity of RAGETag required to fall within the linear range of PCR and for normalization of one sample to another before performing differential expression analysis. There are kits for Human, Mouse and Rat species. A separate kit is required for standardization of A/B or B/A orientation RAGETags. Sufficient reagents are provided to perform 150 PCR reactions per primer set. Reagents should be stored at  $-20^{\circ}\text{C}$  in a non-frost-free freezer. All components may be purchased separately using the product codes listed below.

### Human Standardization Kits

#### **A/B Standardization Kit**

**Product Code: 801-0004**

<u>Kit Component</u>	<u>Product Code</u>	<u>Volume</u>	<u>Amplimer Size (bp)</u>
5X PCR Buffer	800-0013	2 X 1.7 mL	
Human L15 Primer Set	800-0035	300 uL	175
Human L31 Primer Set	800-0036	300 uL	253
Human S11 Primer Set	800-0037	300 uL	67
Human S6 Primer Set	800-0038	300 uL	596
Human L6 Primer Set	800-0039	300 uL	277
Human S18 Primer Set	800-0040	300 uL	105
Human L12 Primer Set	800-0041	300 uL	108
Human L26 Primer Set	800-0042	300 uL	241
Human L19 Primer Set	800-0043	300 uL	163
Human L24 Primer Set	800-0044	300 uL	64

#### **B/A Standardization Kit**

**Product Code: 801-0005**

<u>Kit Component</u>	<u>Product Code</u>	<u>Volume</u>	<u>Amplimer Size (bp)</u>
5X PCR Buffer	800-0013	2 X 1.7 mL	
Human L3 Primer Set	800-0045	300 uL	261
Human S7 Primer Set	800-0046	300 uL	278
Human L35 Primer Set	800-0047	300 uL	160
Human S29 Primer Set	800-0048	300 uL	158
Human L8 Primer Set	800-0049	300 uL	153
Human S15a Primer Set	800-0050	300 uL	136
Human S26 Primer Set	800-0051	300 uL	110
Human S27 Primer Set	800-0052	300 uL	108
Human b-Actin Primer Set	800-0053	300 uL	163
Human L29 Primer Set	800-0054	300 uL	131

**Mouse Standardization Kits****Mouse A/B Standardization Kit      Product Code: 801-0002**

<b><u>Kit Component</u></b>	<b><u>Product Code</u></b>	<b><u>Volume</u></b>	<b><u>Amplimer Size (bp)</u></b>
5X PCR Buffer	800-0013	2 X 1.7 mL	
Mouse S11 Primer Set	800-0015	300 uL	67
Mouse L5 Primer Set	800-0016	300 uL	130
Mouse L3 Primer Set	800-0017	300 uL	68
Mouse L27 Primer Set	800-0018	300 uL	43
Mouse PO Primer Set	800-0019	300 uL	61
Mouse A52 Primer Set	800-0020	300 uL	103
Mouse L29 Primer Set	800-0021	300 uL	295
Mouse g-Actin Primer Set	800-0022	300 uL	93
Mouse S18 Primer Set	800-0023	300 uL	52
Mouse S2 Primer Set	800-0024	300 uL	297

**Mouse B/A Standardization Kit      Product Code: 801-0003**

<b><u>Kit Component</u></b>	<b><u>Product Code</u></b>	<b><u>Volume</u></b>	<b><u>Amplimer Size (bp)</u></b>
5X PCR Buffer	800-0013	2 X 1.7 mL	
Mouse L8 Primer Set	800-0025	300 uL	304
Mouse S26 Primer Set	800-0026	300 uL	110
Mouse L36 Primer Set	800-0027	300 uL	70
Mouse P1 Primer Set	800-0028	300 uL	268
Mouse S4 Primer Set	800-0029	300 uL	132
Mouse L12 Primer Set	800-0030	300 uL	80
Mouse b-Actin Primer Set	800-0031	300 uL	176
Mouse L26 Primer Set	800-0032	300 uL	58
Mouse S17 Primer Set	800-0033	300 uL	108
Mouse GAPDH Primer Set	800-0034	300 uL	279

## Rat Standardization Kits

### **Rat A/B Standardization Kit      Product Code: 801-0006**

<b><u>Kit Component</u></b>	<b><u>Product Code</u></b>	<b><u>Volume</u></b>	<b><u>Amplimer Size (bp)</u></b>
5X PCR Buffer	800-0013	2 X 1.7 mL	
Rat S23 Primer Set	800-0055	300 uL	125
Rat L24 Primer Set	800-0056	300 uL	64
Rat S10 Primer Set	800-0057	300 uL	148
Rat L5 Primer Set	800-0058	300 uL	236
Rat L13a Primer Set	800-0059	300 uL	96
Rat L18 Primer Set	800-0060	300 uL	226
Rat S12 Primer Set	800-0061	300 uL	70
Rat L41 Primer Set	800-0062	300 uL	234

### **Rat B/A Standardization Kit      Product Code: 801-0007**

<b><u>Kit Component</u></b>	<b><u>Product Code</u></b>	<b><u>Volume</u></b>	<b><u>Amplimer Size (bp)</u></b>
5X PCR Buffer	800-0013	2 X 1.7 mL	
Rat PO Primer Set	800-0063	300 uL	147
Rat L30 Primer Set	800-0064	300 uL	109
Rat L21 Primer Set	800-0065	300 uL	165
Rat L6 Primer Set	800-0066	300 uL	88
Rat L10a Primer Set	800-0067	300 uL	109
Rat S27a Primer Set	800-0068	300 uL	292
Rat S26 Primer Set	800-0069	300 uL	110
Rat L10 Primer Set	800-0070	300 uL	85
Rat L36 Primer Set	800-0071	300 uL	70
Rat L32 Primer Set	800-0072	300 uL	76

## 2.3 Additional Required Reagents and Equipment

### 2.3.1 Required Reagents

See Appendix (Section 8) for Recommended Suppliers (Section 8.2) and Reagent Preparation (Section 8.1)

- 10-20 ug total RNA or 2-5 ug poly(A) mRNA
- cDNA Synthesis Kit (Recommended: Invitrogen: cDNA Synthesis Kit or Superscript Choice System or components for cDNA synthesis (see Section 6.2.1))
- Molecular biology grade water
- 10M Ammonium Acetate
- 70% and 100% Ethanol
- TE Buffer
- Taq Polymerase (Recommended: MBI Fermentas Taq (Native or Recombinant), Invitrogen Taq (Native or Recombinant), Invitrogen Platinum Taq, Promega Taq Beads, PE Amplitaq)
- SYBR<sup>®</sup>Green I (BMA), or GelStar<sup>®</sup> (BMA), or Vistra Green (Amersham Pharmacia Biotech)
- 8% TBE Polyacrylamide Gels (available from Invitrogen/Novex and other vendors)
- 0.2 mL sterile PCR tubes or 96 well PCR plates
- 0.6 mL and 1.5 mL low adhesion microcentrifuge tubes (KPL Cat. No. 60-00-54 and 60-00-55)
- Gel Loading Dye (MBI Fermentas, Amresco, others) (Do NOT use Invitrogen's Blue Juice)

### 2.3.2 Required Equipment

- 16°C, 37°C, and 50°C waterbath or incubators
- Microcentrifuge or quick spin minifuge.
- Magnetic particle concentrator (i.e. magnet designed for particle isolation) (KPL Cat . No. 60-00-52)
- Fluorescent Imager with SYBR Green Filter (SynGene Gene Genius, Alpha Innotech, Molecular Devices, Molecular Dynamics)
- Thermocycler
- PAGE apparatus (Invitrogen/Novex and others)

## 3 RAGetag SYNTHESIS PROTOCOL

### 3.1 Detailed Instructions for New Users

#### Notes Before Starting:

- Do not remove restriction enzymes from freezer until just prior to use. Keep on ice while using and immediately return to  $-20^{\circ}\text{C}$  freezer after use.
- Thaw frozen components on ice and briefly spin down in a microcentrifuge (1-2 second pulse spin) prior to use. Keep thawed components on ice at all times.
- Do not place SA-Magnetic beads on ice. Do not freeze SA-Magnetic beads.

#### 3.1.1 Synthesis of 3' Biotinylated cDNA

Any cDNA synthesis system that produces double stranded, full length cDNA can be utilized for GS320. cDNA synthesis kits can be adapted for GS320 by substitution of the random hexamers or oligo(dT) primers with the biotinylated oligo(dT) provided in the RAGetag Synthesis Kit. We recommend the following and provide detailed protocols (Section 6.2).

- Invitrogen cDNA Synthesis Kit (Cat. no. 18267-013) or Invitrogen Superscript Choice System (Cat. No. 18090-019).
- Protocol and Recipes for using a variety of reverse transcriptase enzymes.
- We strongly recommend the use of KPL's cDNA Integrity Kit (Catalog #60-06-00) to verify full-length and intact cDNA before proceeding with the synthesis of RAGetags.

#### 3.1.2 Preparation of A/B RAGetag: Detailed Protocol

##### A. Streptavidin-Magnetic Bead Preparation

1. Remove vial of magnetic beads from refrigerator. The beads will appear as a brown pellet at the bottom of the tube, with clear liquid above the pellet. Resuspend the beads by gently flicking the tube several times.
2. For each sample being processed, remove 100  $\mu\text{L}$  of suspended beads and place into a separate 0.6 mL low adhesion microfuge tube. Place tubes into magnet holder and allow beads to bind to magnet for about 30 seconds. Binding is complete when the liquid in the tube is clear and a brown tinge is no longer apparent in the supernatant.
3. Using a 100  $\mu\text{L}$  or 200  $\mu\text{L}$  pipettor, and with the tube still in the magnet holder, carefully insert the pipet tip into the bottom of the tube and remove the supernatant. Look at the pipette tip as liquid is drawn into it. If any brown color is drawn into the tip replace the material into the tube and allow the magnetic particles to be drawn to the magnet until the supernatant is cleared of beads. Withdraw and discard the supernatant.
4. For each sample being processed, dilute 310  $\mu\text{L}$  of 2X Binding Buffer with an equal volume of molecular biology grade water to create 1X Binding Buffer.
5. Remove tube from magnet and resuspend beads by adding 200  $\mu\text{L}$  of 1X Binding Buffer (1X BB) and gently pipetting up and down. Return tube to magnet holder for 30 seconds or as long as needed to clear the supernatant of beads.
6. Remove supernatant as described in step 3. Repeat wash with 200  $\mu\text{L}$  1X BB twice more for a total of three washes in 1X BB.
7. After the last wash, discard 1X BB and remove tube from the magnet.

## B. Binding of cDNA to Streptavidin Coated Magnetic Beads

- For each RAGetag being generated, dilute 1-1.5 ug of cDNA into Binding Buffer:
 

70 uL	2X Binding Buffer
variable	(1-1.5 ug cDNA)
<u>variable</u>	<u>molecular biology grade water</u>
<b>140 uL</b>	<b>final volume</b>
- Add each diluted cDNA sample to each tube of washed beads. Mix contents by flicking the tube.
- Incubate at room temperature for 30 to 40 minutes with occasional gentle mixing to keep beads suspended. Binding time can be increased to 1 hour without adverse effects.
- During incubation step B3, prepare 700 uL of 1X *Hsp92II* Buffer for each sample to be digested for use in Section C. To prepare 1X *Hsp92II* Buffer dilute 70 uL of 10X *Hsp92II* Buffer into 630 uL molecular biology grade water and mix well by flicking the tube. Pulse spin tube in microcentrifuge 1-2 seconds. Set aside until needed.
- Place tube containing cDNA bound to beads from step B3 into magnet holder and allow beads to bind to magnet for at least 30 seconds.
- Remove supernatant as in section A3 and discard.

## C. *Hsp92II* Digest

- Remove each tube from the magnet and gently resuspend beads in 200 uL of 1X *Hsp92II* Buffer (prepared in Section B4) by pipetting up and down. Return tube to magnet and allow beads to bind to magnet for about 30 seconds.
- Remove supernatant carefully as described in Section A3. Repeat wash with 200 uL 1X *Hsp92II* Buffer twice more for a total of three washes.
- After the last wash, discard supernatant and remove tube from magnet. Gently resuspend washed beads and bound cDNA in 93 uL of 1X *Hsp92II* Buffer. Pipette up and down a few times.
- Add:
 

2 uL	100X BSA
5 uL	<i>Hsp92II</i> enzyme
- Mix well by gently pipetting the contents of the tube up and down. Incubate 1 hour at 37°C.

## D. *DpnII* Digest

- For each sample, prepare 700 uL of 1X *DpnII* Buffer by diluting 70 uL of 10X *DpnII* Buffer into 630 uL of molecular biology grade water and mix well by flicking the tube. Pulse spin tube in microcentrifuge 5 seconds. Set aside until needed.
- Pulse-spin tube from the *Hsp92II* restriction digestion (Section C5) 1-2 seconds.
- Add 200 uL of 1X *DpnII* Buffer to tube. Resuspend by gently pipetting up and down a few times.
- Place tube into magnet holder for 30 seconds.
- Carefully remove supernatant.
- Remove tube from the magnet holder. Repeat wash step in 1X *DpnII* Buffer twice more for a total of three washes.
- After the last wash in 1X *DpnII* Buffer, discard the wash and remove tube from the magnet holder.
- Gently resuspend washed beads with bound cDNA in 95 uL of 1X *DpnII* Buffer.
- Add 5 uL of *DpnII* enzyme. Mix well by pipetting the contents of the tube up and down.
- Incubate for 1 hour at 37°C.

11. After *DpnII* digest, pulse-spin tube in microcentrifuge 1-2 seconds.
12. Place tube in magnet holder and allow beads to bind for 30 seconds. Refer to Section A2.
13. **DO NOT DISCARD SUPERNATANT** (the supernatant contains the RAGetag sample). Carefully remove supernatant and place it into a 1.5 mL low adhesion microcentrifuge tube. Be careful that only the supernatant is removed from the tube and not beads. Discard the beads.

#### E. PureBind-Bead Precipitation

1. Place 700  $\mu$ L of cold 100% ethanol into a low adhesion 1.5 mL microcentrifuge tube.
2. Flick tube of PureBind Beads until the beads are in suspension.
3. Pipette 70  $\mu$ L of beads into the tube with ethanol. Mix well by inversion. Pulse-spin 1-2 seconds and place tube into magnet.
4. Remove supernatant from PureBind Beads. **Immediately remove tube from magnet and place in holder on benchtop.** Add 700  $\mu$ L of 100% cold ethanol. Mix by inverting the tube until a suspension is formed. Pulse-spin 1-2 seconds.
5. Return tube to magnet and remove supernatant. Repeat wash 1X as above.
6. After last wash, resuspend beads in 700  $\mu$ L 100% ethanol and place on ice.
7. Add 100  $\mu$ L of 1X TE to cDNA from Section D13. Add 3  $\mu$ L glycogen and 100  $\mu$ L 10M Ammonium Acetate. Mix well by inversion. Pulse-spin 1-2 seconds.
8. Add cDNA/glycogen/Ammonium Acetate to beads and mix by inverting the tube. Pulse-spin 1-2 seconds.
9. Place on ice for 15 minutes.
10. Bind to magnet, and remove supernatant.
11. Wash three times with 700  $\mu$ L of cold 70% ethanol. Don't pipette the beads in the ethanol. Mix by inverting tube until a suspension is formed. Pulse-spin 1-2 seconds prior to placing tube in magnet.
12. With the last wash, remove as much 70% ethanol as possible. Remove tube from magnet and spin in microcentrifuge. Return tube to magnet and remove residual 70% ethanol.
13. Dry beads 3-5 minutes at room temperature. Monitor drying carefully-**Do not** let the bead pellet crack or cake in the tube.
14. Resuspend beads in 25  $\mu$ L of 1X TE. Vortex. Let sit at room temperature for 10 minutes. Vortex briefly and pulse-spin 1-2 seconds in microcentrifuge. Bind to magnet and **keep supernatant** containing the RAGetag sample. Discard beads.

#### F. Linker A and B addition

1. Add to tube on ice:
  - 5  $\mu$ L 10 X Ligase Buffer
  - 5  $\mu$ L Biotinylated Linker A
  - 5  $\mu$ L Linker B
2. Mix well and heat to 50°C for 2 minutes. Cool to room temperature for 15 minutes.
3. Add 2  $\mu$ L of T4 DNA Ligase and incubate at room temperature for 15 minutes to 1 hour.

#### G. Final Sample Processing

1. For each sample being processed, dilute 310  $\mu$ L of 2X Binding Buffer with an equal volume of molecular biology grade water to create 1X Binding Buffer.
2. Prepare 100  $\mu$ L of SA-magnetic beads by immobilizing slurry on magnet. Remove supernatant. Wash with 200  $\mu$ L of 1X BB. Repeat wash 2X.

3. Remove last wash and add:
  - 50 uL of ligated DNA (supernatant from step F3).
  - 100 uL of 2X Binding Buffer
  - 50 uL of molecular biology grade water
4. Incubate at room temperature for 30 minutes with intermittent mixing.
5. Immobilize beads on magnet and remove supernatant.
6. Wash the beads 3X in 1X TE.
7. After final wash, resuspend beads in 100 uL of 1X TE and store at 4°C. **DO NOT FREEZE.**
8. Proceed to synthesis of B/A RAGEtag (Section 3.1.3) or Standardization Protocol (Section 4).

### 3.1.3 Preparation of B/A RAGetag: Detailed Protocol

#### A. SA-Magnetic Bead Preparation

1. Remove vial of magnetic beads from refrigerator. The beads will appear as a brown pellet at the bottom of the tube, with clear liquid above the pellet. Resuspend the beads by gently flicking the tube several times.
2. For each sample being processed, remove 100 uL of suspended beads and place into a separate 0.6 mL low adhesion microfuge tube. Place tubes into magnet holder and allow beads to bind to magnet for about 30 seconds. Binding is complete when the liquid in the tube is clear and a brown tinge is no longer apparent in the supernatant.
3. Using a 100 uL or 200 uL pipettor, and with the tube still in the magnet holder, carefully insert the pipet tip into the bottom of the tube and remove supernatant. Look at the pipette tip as liquid is drawn into it. If any brown color is drawn into the tip replace the material into the tube and allow the magnetic particles to be drawn to the magnet until the supernatant is cleared of beads. Withdraw and discard the supernatant.
4. For each sample being processed prepare 1X Binding Buffer by diluting 310 uL of 2X Binding Buffer with an equal volume of molecular biology grade water.
5. Remove tube from magnet and resuspend beads by adding 200 uL of 1X Binding Buffer (1X BB) and gently pipetting up and down. Return tube to magnet holder for 30 seconds or as long as is needed to clear the supernatant of beads.
6. Remove supernatant as described in step A3. Repeat wash with 200 uL 1X BB twice more for a total of three washes in 1X BB.
7. After the last wash, discard 1X BB and remove tube from the magnet.

#### B. Binding of cDNA to Streptavidin Coated Magnetic Beads

1. For each RAGetag being generated, dilute 1-1.5 ug of cDNA into Binding Buffer:
2. Add:
 

70 uL	2X Binding Buffer
variable	1-1.5 ug cDNA
variable	<u>molecular biology grade water</u>
<b>140 uL</b>	<b>final volume</b>
3. Add each diluted cDNA sample to each tube of washed beads. Mix well by gently pipetting up and down.
4. Incubate at room temperature for 30 to 40 minutes with occasional gentle mixing to keep beads suspended. Binding time can be increased to 1 hour without adverse effects.
5. During incubation step B3 prepare 700 uL of 1X *DpnII* Buffer for each sample to be digested for use in Section C. To prepare 1X *DpnII* Buffer dilute 70 uL of 10X *DpnII* Buffer into 630 uL molecular biology grade water and mix well by flicking the tube. Pulse spin tube in microcentrifuge 5 seconds. Set aside until needed.
6. Place tube containing cDNA bound to beads from step B3 into magnet holder and allow beads to bind to magnet for at least 30 seconds.
7. Remove supernatant as in Section A3 and discard.

### C. *DpnII* Digest

1. Remove the tube from the magnet holder and gently resuspend beads in 200  $\mu$ L of 1X *DpnII* buffer by pipetting up and down. Return the tube to magnet holder and allow beads to bind to magnet for about 30 seconds.
2. Remove supernatant carefully as described in Section A3. Repeat wash with 200  $\mu$ L of 1X *DpnII* Buffer twice more for a total of three washes.
3. After the last wash, discard supernatant and remove tube from magnet holder. Gently resuspend washed beads and bound cDNA in 95  $\mu$ L of 1X *DpnII* Buffer. Pipette up and down a few times.
4. Add 5  $\mu$ L of *DpnII* enzyme.
5. Mix well by gently pipetting the contents of the tube up and down. Incubate for 1 hour at 37°C.

### D. *Hsp92II* Digest

1. For each sample, prepare 700  $\mu$ L of 1X *Hsp92II* Buffer by diluting 70  $\mu$ L of 10X *Hsp92II* Buffer into 630  $\mu$ L of molecular biology grade water and mix well by flicking the tube. Pulse spin tube in microcentrifuge 1-2 seconds. Set aside until needed.
2. Pulse-spin tube from the *DpnII* digestion (Section C5) in a microcentrifuge 1-2 seconds.
3. Add 200  $\mu$ L of 1X *Hsp92II* Buffer to tube. Resuspend gently by pipetting up and down a few times.
4. Place tube into magnet holder for about 30 seconds. Carefully remove supernatant.
5. Remove tube from the magnet holder. Repeat wash step in 1X *Hsp92II* Buffer twice more for a total of three washes.
6. After the last wash in 1X *Hsp92II* Buffer, discard the wash and remove tube from the magnet holder.
7. Gently resuspend washed beads with bound cDNA in 93  $\mu$ L of 1X *Hsp92II* Buffer. Pipette up and down a few times.
8. Add:
  - 2  $\mu$ L 100X BSA.
  - 5  $\mu$ L *Hsp92II* enzyme
9. Mix well by gently pipetting the contents of the tube up and down.
10. Incubate for 1 hour at 37°C.
11. After *Hsp92II* digest, pulse-spin tube in microcentrifuge 1-2 seconds.
12. Place tube in magnet holder and allow beads to bind for about 30 seconds.
13. **DO NOT DISCARD SUPERNATANT** (the supernatant contains your RAGetag sample). Carefully remove supernatant and place it into a 1.5 mL low adhesion microcentrifuge tube. Be careful that only the supernatant is removed from the tube and not beads. Discard the beads.

### E. PureBind Bead Precipitation

1. Place 700  $\mu$ L of cold 100% ethanol into a low adhesion 1.5 mL microcentrifuge tube.
2. Flick tube of PureBind beads until the beads are in suspension.
3. Pipette 70  $\mu$ L of beads into the tube with ethanol. Mix well by inversion. Pulse-spin 1-2 seconds and place tube into magnet.
4. Remove supernatant from PureBind Beads. **Immediately remove tube from magnet and place in holder on benchtop.** Add 700  $\mu$ L of 100 % cold ethanol. Mix by inverting the tube until a suspension is formed. Pulse-spin 1-2 seconds.
5. Return tube to magnet and remove supernatant. Repeat wash 1X as above.
6. After last wash, resuspend beads in 700  $\mu$ L 100 % ethanol and place on ice.

7. Add 100  $\mu$ L of 1X TE to cDNA from Section D13. Add 3  $\mu$ L glycogen and 100  $\mu$ L 10M Ammonium Acetate. Mix well by inversion. Pulse-spin 1-2 seconds.
8. Add cDNA/glycerol/ammonium acetate solution to beads and mix by inverting the tube. Pulse-spin 1-2 seconds.
9. Place on ice for 15 minutes.
10. Bind to magnet, and remove supernatant.
11. Wash three times with 700  $\mu$ L of cold 70% ethanol. Don't pipette the beads in the ethanol. Mix by inverting tube until a suspension is formed. Pulse-spin 1-2 seconds prior to placing tube in magnet.
12. With the last wash, remove as much 70% ethanol as possible. Remove tube from magnet and spin in microcentrifuge. Return tube to magnet and remove residual 70% ethanol.
13. Dry beads 3-5 minutes at room temperature. Monitor drying carefully-Do not let the bead pellet crack or cake in the tube.
14. Resuspend beads in 25  $\mu$ L of 1X TE. Vortex. Let sit at room temperature for 10 minutes. Vortex briefly and pulse-spin 1-2 seconds in microcentrifuge. Bind to magnet and **keep supernatant** containing the RAGetag sample. Discard beads.

#### F. Linker A and B addition

1. Add to tube on ice:
  - 5  $\mu$ L 10 X Ligase Buffer
  - 5  $\mu$ L Biotinylated Linker B
  - 5  $\mu$ L Linker A
2. Mix well and heat to 50°C for 2 minutes. Cool to room temperature for 15 minutes.
3. Add 2  $\mu$ L of T4 DNA Ligase and incubate at room temperature for 15 minutes to 1 hour.

#### G. Final Sample Processing

1. For each sample being processed, dilute 310  $\mu$ L of 2X Binding Buffer with an equal volume of molecular biology grade water to create 1X Binding Buffer.
2. Prepare 100  $\mu$ L of SA-magnetic beads by immobilizing slurry on magnet. Remove supernatant. Wash with 200  $\mu$ L of 1X BB. Repeat wash 2X.
3. Remove last wash and add:
  - 50  $\mu$ L of ligated DNA (supernatant from step F3).
  - 100  $\mu$ L of 2X Binding Buffer
  - 50  $\mu$ L of molecular biology grade water
4. Incubate at room temperature for 30 minutes with intermittent mixing.
5. Immobilize beads on magnet and remove supernatant.
6. Wash the beads 3X in 1X TE.
7. After final wash, resuspend beads in 100  $\mu$ L of 1X TE and store at 4°C. **DO NOT FREEZE.**
8. Proceed to Standardization Protocol (Section 4).

## 3.2 Brief Protocol for Experienced Users

Note: The RAGEtag Synthesis protocol on the next four pages may be photocopied and used as a worksheet to keep track of your progress throughout the procedure by checking off steps as they are completed.

### 3.2.1 Synthesis of 3' Biotinylated cDNA

Any cDNA synthesis system that produces double stranded, full length cDNA can be utilized for GS320. cDNA synthesis kits can be adapted for GS320 by substitution of the random hexamers or oligo(dT) primers with the biotinylated oligo(dT) provided in the RAGEtag Synthesis Kit. We recommend the following and provide detailed protocols in Section 6.2:

- Invitrogen cDNA Synthesis Kit (Cat. no. 18267-013) or Superscript Choice System (Cat. No. 18090-019).
- Protocol and Recipes for use of a variety of Reverse Transcriptase enzymes.
- We strongly recommend the use of KPL's cDNA Integrity Kit (Catalog #60-06-00) to verify full-length and intact cDNA before proceeding with the RAGEtag synthesis protocol.

### 3.2.2 Preparation of A/B RAGetag: Short Protocol

#### A. Bind cDNA to SA-Magnetic beads

1. For each RAGetag being generated, dilute 1-1.5 ug of cDNA into Binding Buffer as follows in a low adhesion microfuge tube:

70 uL	2X Binding Buffer (BB)
variable	1-1.5 ug cDNA
variable	molecular biology grade water
<b>140 uL</b>	<b>final volume</b>

2. Prepare 100 uL of SA-magnetic beads by immobilizing slurry on magnet. Remove supernatant. Wash with 200 uL of 1X BB. Repeat wash 2X.
3. Remove last wash.
4. Add the 140 uL of diluted cDNA to beads. Incubate at room temperature for 30-40 minutes with intermittent mixing.
5. Immobilize beads on magnet and remove supernatant.

#### B. *Hsp92II* digestion

1. Wash beads with 200 uL of 1X *Hsp92II* buffer. Repeat 2X.
2. Remove last wash. Add:
- |       |                          |
|-------|--------------------------|
| 93 uL | 1X <i>Hsp92II</i> buffer |
| 5 uL  | <i>Hsp92II</i> enzyme    |
| 2 uL  | 100X BSA                 |

3. Incubate at 37°C for 1 hr.
4. Immobilize beads on magnet and remove supernatant.

#### C. *DpnII* digestion

1. Add 200 uL of 1X *DpnII* buffer. Immobilize beads and remove supernatant. Repeat 2X. Remove last wash.
2. Add:
- |       |                        |
|-------|------------------------|
| 95 uL | 1X <i>DpnII</i> buffer |
| 5 uL  | <i>DpnII</i> enzyme    |

3. Incubate at 37°C for 1 hour.
4. Immobilize beads. Remove and **KEEP SUPERNATANT** in a 1.5 mL low adhesion tube. Discard beads.

#### D. PureBind Bead Precipitation

1. Place 700 uL of cold 100% ethanol into a low adhesion 1.5 mL microcentrifuge tube.
2. Flick tube of PureBind Beads until the beads are in suspension.
3. Pipette 70 uL of beads into the tube with ethanol. Mix well by inversion. Pulse-spin 1-2 seconds and place tube into magnet.
4. Remove supernatant. **Immediately remove tube from magnet and place in holder on benchtop.** Add 700 uL of 100 % cold ethanol. Mix by inverting the tube until a suspension is formed. Centrifuge briefly.
5. Return tube to magnet and remove supernatant. Repeat wash 1X as above.
6. After last wash, resuspend beads in 700 uL 100 % ethanol and place on ice.

- 7. Add 100 uL of 1X TE to cDNA from Section C4. Add 3 uL glycogen and 100 uL 10M Ammonium Acetate. Mix well by inversion. Pulse-spin 1-2 seconds. Add cDNA solution to beads and mix by inverting the tube. Pulse-spin 1-2 seconds.
- 8. Place on ice for 15 minutes.
- 9. Bind to magnet, and remove supernatant.
- 10. Wash three times with 700 uL of cold 70% ethanol. Don't pipette the beads in the ethanol. Mix by inverting tube until a suspension is formed. Pulse-spin 1-2 seconds prior to placing tube in magnet.
- 11. With the last wash, remove as much 70% ethanol as possible. Remove tube from magnet and pulse-spin 1-2 seconds in microcentrifuge. Return tube to magnet and remove residual 70% ethanol.
- 12. Dry beads 3-5 minutes at room temperature. Monitor drying carefully-**Do not** let the bead pellet crack or cake in the tube.
- 13. Resuspend beads in 25 uL of 1X TE. Vortex. Let sit at room temperature 10 minutes. Vortex briefly and pulse-spin 1-2 seconds in microcentrifuge. Bind to magnet and **keep supernatant** with the RAGEtag sample. Discard beads.

### E. Linker A and B addition

- 1. Add to tube on ice:
  - 5 uL 10 X Ligase Buffer
  - 5 uL Biotinylated Linker A
  - 5 uL Linker B
- 2. Mix well and heat to 50°C for 2 minutes. Cool to room temperature for 15 minutes.
- 3. Add 2 uL of T4 DNA Ligase and incubate at room temperature for 15 minutes to 1 hour.

### F. Final Sample Processing

- 1. Prepare 100 uL of SA-magnetic beads by immobilizing slurry on magnet. Remove supernatant. Wash with 200 uL of 1X BB. Repeat wash 2X.
- 2. Remove last wash and add:
  - 50 uL of ligated DNA (supernatant from step E3).
  - 100 uL of 2X Binding Buffer
  - 50 uL of molecular biology grade water
- 3. Incubate at room temperature for 30 minutes with intermittent mixing.
- 4. Immobilize beads on magnet and remove supernatant.
- 5. Wash the beads 3X in 1X TE.
- 6. After final wash, resuspend beads in 100 uL of 1X TE and store at 2-8°C. **DO NOT FREEZE.**

### 3.2.3 Preparation of B/A RAGetag: Short Protocol

#### A. Bind cDNA to SA-Magnetic beads

1. For each RAGetag being generated, dilute 1-1.5 ug of cDNA into Binding Buffer as follows in a low adhesion microfuge tube:

70 uL	2X Binding Buffer (BB)
variable	1-1.5 ug cDNA
variable	molecular biology grade water
<b>140 uL</b>	<b>final volume</b>

2. Prepare 100 uL of SA-magnetic beads by immobilizing slurry on magnet. Remove supernatant. Wash with 200 uL of 1X BB. Repeat wash 2X.
3. Remove last wash.
4. Add the 140 uL of diluted cDNA to beads. Incubate at room temperature for 30-40 minutes with intermittent mixing.
5. Immobilize beads on magnet and remove supernatant.

#### B. *DpnII* digestion

1. Wash beads with 200 uL of 1X *DpnII* buffer. Repeat 2X.
2. Remove last wash. Add:
- |       |                        |
|-------|------------------------|
| 95 uL | 1X <i>DpnII</i> buffer |
| 5 uL  | <i>DpnII</i> enzyme    |
3. Incubate at 37°C for 1 hr.
4. Immobilize beads on magnet and remove supernatant.

#### C. *Hsp92II* digestion

1. Wash beads with 200 uL of 1X *Hsp92II* buffer. Repeat 2X.
2. Remove last wash. Add:
- |       |                          |
|-------|--------------------------|
| 93 uL | 1X <i>Hsp92II</i> buffer |
| 5 uL  | <i>Hsp92II</i> enzyme    |
| 2 uL  | 100X BSA                 |
3. Incubate at 37°C for 1 hr.
4. Immobilize beads. Remove and **KEEP SUPERNATANT** in a 1.5 mL low adhesion tube. Discard beads.

#### D. PureBind Bead Precipitation

1. Place 700 uL of cold 100% ethanol into a low adhesion 1.5 mL microcentrifuge tube.
2. Flick tube of PureBind beads until the beads are in suspension.
3. Pipette 70 uL of beads into the tube with ethanol. Mix well by inversion. Pulse-spin 1-2 seconds and place tube into magnet.
4. Remove supernatant. **Immediately remove tube from magnet and place in holder on benchtop.** Add 700 uL of 100 % cold ethanol. Mix by inverting the tube until a suspension is formed. Pulse-spin 1-2 seconds.
5. Return tube to magnet and remove supernatant. Repeat wash 1X as above.
6. After last wash, resuspend PureBind beads in 700 uL 100 % ethanol and place on ice.

- 7. Add 100 uL of 1X TE to supernatant. Add 3 uL glycogen and 100 uL 10M Ammonium Acetate. Mix well by inversion. Centrifuge briefly. Add DNA solution to beads and mix by inverting the tube. Centrifuge briefly.
- 8. Place on ice for 15 minutes.
- 9. Bind to magnet, and remove supernatant.
- 10. Wash three times with 700 uL of cold 70% ethanol. Don't pipette the beads in the ethanol. Mix by inverting tube until a suspension is formed. Pulse-spin 1-2 seconds prior to placing tube in magnet.
- 11. With the last wash, remove as much 70% ethanol as possible. Remove tube from magnet and pulse-spin 1-2 seconds in microcentrifuge. Return tube to magnet and remove residual 70% ethanol.
- 12. Dry beads 3 minutes at room temperature. Monitor drying carefully-**Do not** let the bead pellet crack or cake in the tube.
- 13. Resuspend beads in 25 uL of 1X TE. Vortex. Let sit at room temperature 10 minutes. Vortex briefly and pulse-spin 1-2 seconds in microcentrifuge. Bind to magnet and **keep supernatant** with the RAGEtag sample. Discard beads.

#### E. Linker A and B addition

- 1. Add to tube on ice:
  - 5 uL 10 X Ligase Buffer
  - 5 uL Linker A
  - 5 uL Biotinylated Linker B
- 2. Mix well and heat to 50°C for 2 minutes. Cool to room temperature for 15 minutes.
- 3. Add 2 uL of T4 DNA Ligase and incubate at room temperature for 15 minutes to 1 hour.

#### F. Final Sample Processing

- 1. Prepare 100 uL of SA-magnetic beads by immobilizing slurry on magnet. Remove supernatant. Wash with 200 uL of 1X BB. Repeat wash 2X.
- 2. Remove last wash and add:
  - 50 uL of ligated DNA (supernatant from step E3).
  - 100 uL of 2X Binding Buffer
  - 50 uL of molecular biology grade water
- 3. Incubate at room temperature for 30 minutes with intermitent mixing.
- 4. Immobilize beads on magnet and remove supernatant.
- 5. Wash the beads 3X in 1X TE.
- 6. After final wash, resuspend beads in 100 uL of 1X TE and store at 2-8°C. **DO NOT FREEZE.**

## 4 GS320 STANDARDIZATION PROTOCOL

### 4.1 Survey of Standardization Gene Expression

The GS320 protocol results in semi-quantitative data. The GS320 5X PCR buffer and the reaction conditions are in excess and therefore allow exponential accumulation of product. PCR products will accumulate linearly with increasing template concentration as long as the template is limiting. It is therefore necessary to determine the optimal concentration of RAGetag necessary to maintain a constant rate of PCR product synthesis. This is accomplished by titrating the amount of RAGetag used in PCR reactions. Perform the PCR reaction with the appropriate primer pairs supplied in the standardization kits.

Not all of the standardization genes may be expressed in every system. When first analyzing a model system, utilize all the standardization genes in the kit. Determine which of the genes are expressed in the model system and then utilize only that subset of genes for subsequent experiments.

The genes supplied in the standardization kit were chosen for the following traits:

- They are constitutively expressed in a wide variety of test systems.
- Genes are expressed at low, moderate and abundant levels of expression.
- They generate amplicons of varying lengths.

#### Notes Before Starting

- It is not necessary to perform the linearity assay on all your samples. We recommend use of the untreated or control sample as that is usually greatest in abundance.
- Thaw frozen components on ice. Pulse-spin components in microcentrifuge to bring all material to the bottom of the tube. Place on ice until needed.
- We recommend establishing a “clean area” for PCR set-up. Use of a PCR Hood for sample set-up is optimal.

#### 4.1.1 PCR Reaction Set up

1. Prepare a 1:250 dilution of one RAGetag preparation in TE Buffer (1 uL of RAGetag in 249 uL of TE Buffer).
2. Prepare reactions containing 1.0 uL of the diluted RAGetag preparation for analysis of each standardization gene. Run duplicate samples for each standardization gene.
3. When performing multiple assays, use of a master mix is recommended. Prepare the reaction master mixture for triplicate samples in sterile tubes. Set reactions up on ice. Use molecular biology grade water.
4. Use the following as a guide for setting up a master mix (N = the number of standardization genes being assayed plus one extra reaction to account for pipettor error). The template below is set up for triplicate samples.

Component	Volume/rxn	1 uL
<b>RAGetag</b>	1 uL	2 uL x (N +1)
<b>5X PCR Buffer</b>	5 uL	10 uL x (N +1)
<b>Taq Pol (1.25 U)</b>	Variable	__ uL x (N +1)
<b>Water*</b>	Variable	__ uL x (N +1)
<b>Total</b>	<b>23 uL</b>	<b>46 uL x (N +1)</b>

\*molecular biology grade

5. For each standardization gene being assayed, aliquot 23 uL of the master mix into two PCR tubes on ice.
6. Add 2 uL of the primer set for the appropriate gene to the tubes. Pulse-spin down tubes. Perform PCR using the conditions in the table below.

#### 4.1.2 PCR Cycling Conditions

Condition	Time	Temperature	Cycles
Denaturation	5 minutes	95°C	1 cycle
Denaturation	30 seconds	95°C	28 cycles
Anneal	1 minute	60°C	
Extend	1 minute	72°C	
Final Extension	6 minutes	72°C	1 cycle
Refrigerate	Hold	4°C	

#### 4.1.3 Gel Analysis

1. Run 10 uL of each PCR reaction and the appropriate volume of DNA loading dye on a polyacrylamide gel. We recommend 8% polyacrylamide/TBE gels. Use of gels in large format (16 cm-20 cm plate size) allow for many samples to be run on the same gel, which makes quantitation of the signal easier than using many small format gels.
2. After electrophoresis, soak each gel for approximately 30-60 seconds in a 1:10,000 dilution of SYBR Green I, Vistra Green or GelStar diluted in 1X TBE. The same preparation of diluted fluorescent dye may be used to stain multiple gels, but do not store and reuse diluted dye after initial use.
3. Determine which standardization genes are expressed in your system based on the fluorescent intensity of the PCR amplicon and the size of the fragment. Choose one low and one moderate to highly expressed gene to determine the linear range of PCR (Section 4.2).

## 4.2 Determination of the Linear Range of PCR

### 4.2.1 PCR Reaction Set up

1. Prepare a 1:250 dilution of one RAGEtag preparation in TE Buffer (1 uL of RAGEtag in 249 uL of TE Buffer).
2. Prepare reactions containing 0.5, 1.0, 2.0, 4.0 and 8.0 uL of the diluted RAGEtag preparation for analysis of each standardization gene. Run triplicate samples of each dilution for each standardization gene.
3. When performing multiple assays, use of a master mix is recommended. Prepare the reaction master mixture for triplicate samples in sterile tubes. Set reactions up on ice. Use molecular biology grade water.

4. Use the following as a guide for setting up a master mix (N = the number of standardization genes being assayed plus one extra reaction to account for pipettor error). The template below is set up for triplicate samples.

	<b>Volume /rxn</b>	<b>0.5 uL</b>	<b>1 uL</b>	<b>2 uL</b>	<b>4 uL</b>	<b>8 uL</b>
<b>RAGEtag</b>	Variable	1.5 uL x (N +1)	3 uL x (N +1)	6 uL x (N +1)	12 uL x (N +1)	24 uL x (N +1)
<b>5X PCR Buffer</b>	5 uL	15 uL x (N +1)	15 uL x (N +1)	15 uL x (N +1)	15 uL x (N +1)	15 uL x (N +1)
<b>Taq Pol (1.25 U)</b>	Variable	__ uL x (N +1)	__ uL x (N +1)	__ uL x (N +1)	__ uL x (N +1)	__ uL x (N +1)
<b>Water*</b>	Variable	__ uL x (N +1)	__ uL x (N +1)	__ uL x (N +1)	__ uL x (N +1)	__ uL x (N +1)
<b>Total</b>	<b>23 uL</b>	<b>69 uL x (N +1)</b>	<b>69 uL x (N +1)</b>	<b>69 uL x (N +1)</b>	<b>69 uL x (N +1)</b>	<b>69 uL x (N +1)</b>

\*molecular biology grade

5. For each standardization gene being assayed, aliquot 23 uL of the master mix into three PCR tubes on ice. Repeat for each dilution of RAGEtag.
6. Add 2 uL of the primer set for the appropriate gene to the tubes. Pulse-spin down tubes. Perform PCR using the conditions in the table below.

#### 4.2.2 PCR Cycling Conditions

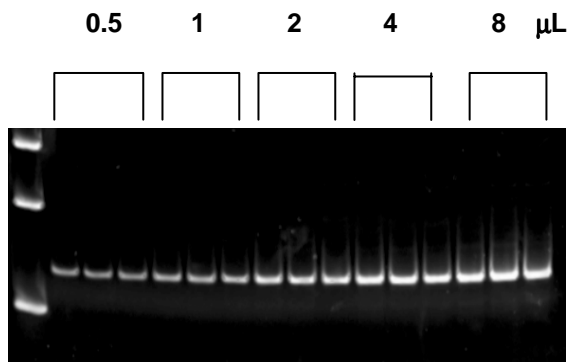
<b>Condition</b>	<b>Time</b>	<b>Temperature</b>	<b>Cycles</b>
<b>Denaturation</b>	5 minutes	95°C	1 cycle
<b>Denaturation</b>	30 seconds	95°C	28 cycles
<b>Anneal</b>	1 minute	60°C	
<b>Extend</b>	1 minute	72°C	
<b>Final Extension</b>	6 minutes	72°C	1 cycle
<b>Refrigerate</b>	Hold	4°C	

#### 4.2.3 Gel Analysis

7. Run 10 uL of each PCR reaction and the appropriate volume of DNA loading dye on a polyacrylamide gel. We recommend 8% polyacrylamide/TBE gels. Use of gels in large format (16 cm-20 cm plate size) allow for many samples to be run on the same gel, which makes quantitation of the signal easier than using many small format gels.
8. After electrophoresis, soak each gel for approximately 30-60 seconds in a 1:10,000 dilution of SYBR Green I, Vistra Green or GelStar diluted in 1X TBE. The same preparation of diluted fluorescent dye may be used to stain multiple gels, but do not store and reuse diluted dye after initial use.

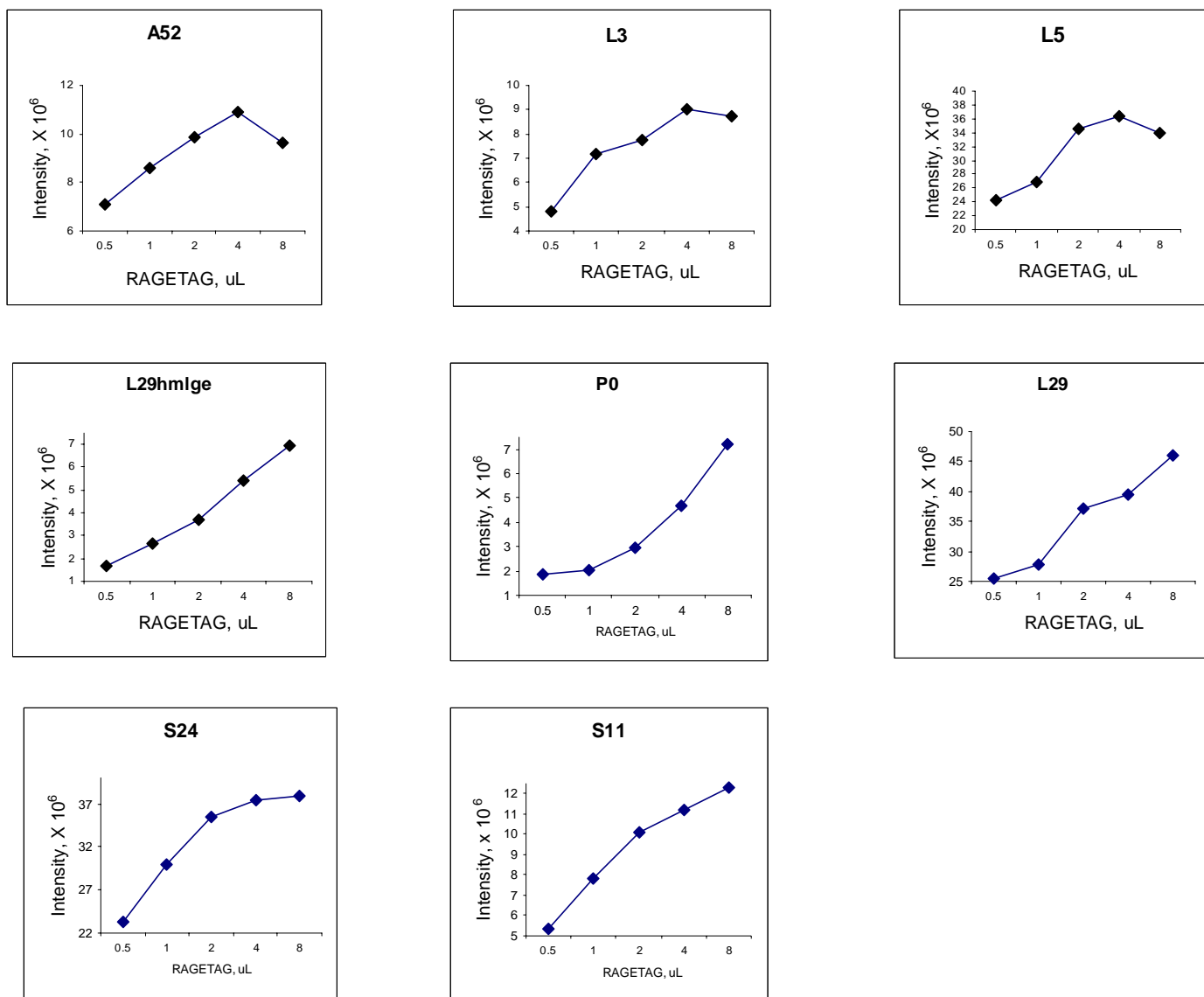
#### 4.2.4 Visualization on a fluorescent imager.

Example:



**Figure 1.** B/A RAGEtags were prepared from WEHI-231 B-cell Lymphoma cells according to the standard protocol. The linear range of PCR was determined by titration of one RAGEtag sample using one of the standardization genes. Triplicate samples of each dilution were analyzed. The gel was stained in SYBR<sup>TM</sup> Green I and visualized on a Fluorescent Imager.

1. Perform densitometric analysis of each band following the protocol for each specific fluorescent imager. Calculate the average of the densitometric value generated from the fluorescent signal for the triplicate samples.
2. Plot the averaged densitometry values (y axis) against the volume of a 1:250 dilution of RAGEtag sample used (x axis). An example of a linearity experiment using 8 primer pairs is shown in Figure 2.



**Figure 2.** Plots of linearity experiment using 8 different primer pairs. RAGETag was titrated into PCR reactions that contained the primer pairs that amplify standardization genes. From these plots it is possible to determine the amount of RAGETag to use for subsequent experiments such as normalization of samples and analysis of specific genes. Visual analysis from the plots above indicate that 2 uL of RAGETag is optimal to maintain a constant rate of PCR product synthesis for all of the standardization genes.

3. From the plots determine the range where the amount of product generated during PCR is proportional to the increase linearly with increasing input material. Perform the analysis with all of the standardization genes expressed in your system to find a dilution where all of the genes fall within the linear range. It may be necessary to repeat the experiment, optimizing the suggested dilution ranges.
4. Proceed to Normalization of Samples (Section 4.2).

### 4.3 Normalization of Samples

GS320 utilizes multiple control genes for normalization of one sample to another. Use of multiple control genes for normalization of one sample to another is more reliable than using a single gene to normalize samples to one another. The GS320 Standardization Kits contain a panel of ribosomal protein genes and other housekeeping genes for quantitation of one sample to another.

The standardization genes that were found to be expressed in your system from the previous linearity experiment are used to find at least 4-5 genes that are constitutively expressed in the tissue or cells of interest. Analysis is carried out by performing parallel PCR reactions on the samples with the standardization genes. The densitometric ratio of amplicon produced from one sample relative to others is then used to normalize the concentration of the RAGEtags across all samples.

#### Notes Before Starting

- Thaw frozen components on ice. Spin in microcentrifuge to bring all material to the bottom of the tube. Place on ice until needed.
- We recommend establishing a “clean area” for PCR set-up. Use of a PCR Hood for sample set-up is best.
- When normalizing multiple test samples to one control, adjust the volume of the test samples while maintaining a constant volume of the control.

#### 4.3.1 PCR Set Up

1. Set up triplicate PCR reactions for each expressed standardization gene with control and treated samples. Use the amount of RAGEtag determined to be in the linear range of PCR. For example, if the standardization genes were all in the linear range using 2 uL of a 1:250 dilution, then use 2 uL of a 1:250 dilution of control sample and 2 uL of a 1:250 dilution of treated sample for PCR.
2. Use of a master mix is recommended. Use the following as a guide for setting up the master mix. (N = the number of genes). An extra reaction volume of each reagent is included to account for pipettor error.
3. Set up Master Mixes for Control and Treated samples as shown in the table below. Increase volumes as necessary based on the number of genes being assayed. Set up reactions on ice.

Component	Volume/rxn	Amount in uL (triplicate samples)
<b>RAGEtag</b>	Variable	( __ uL x N) + 1 rxn vol. of RAGEtag
<b>5X PCR Buffer</b>	5 uL	(15 uL x N) + 5 uL of PCR Buffer
<b>Taq Pol (1.25 U)</b>	Variable	( __ uL x N) + 1.25 units Taq
<b>Water*</b>	Variable	( __ uL x N) + 1 rxn vol. of water
<b>Total</b>	<b>23 uL</b>	<b>(69 uL x N)+ 23 uL</b>

\*molecular biology grade water

4. For each gene being assayed, aliquot 23 uL of each Master Mix into three PCR tubes on ice.
5. Add 2 uL of primer set for the appropriate standardization gene to each tube. Pulse-spin down tubes.
6. Perform PCR using the conditions given below.

### 4.3.2 PCR Cycling Conditions.

Condition	Time	Temperature	Cycles
Denaturation	5 minutes	95°C	1 cycle
Denaturation	30 seconds	95°C	28 cycles
Anneal	1 minute	60°C	
Extend	1 minute	72°C	
Final Extension	6 minutes	72°C	1 cycle
Refrigerate	Hold	4°C	

### 4.3.3 Gel Analysis

1. Take 10 uL of each PCR reaction, add DNA loading dye and run on an 8% Polyacrylamide gel (16 cm-20 cm plate size).
2. After electrophoresis, soak each gel for approximately 30-60 seconds in a 1/10,000 dilution of SYBR Green I, Vistra Green or GelStar diluted in 1X TBE. The same preparation of diluted fluorescent dye may be used to stain multiple gels, but do not store and reuse diluted dye after initial use. Visualize on a Fluorescent Imager.
3. Obtain a densitometric reading for all the bands, and average the triplicate values for each standardization gene for the control and treated samples.
4. Calculate a Treated/Control (T/C) densitometry ratio for each gene. Use a statistical analysis program, such as Excel, to obtain a median T/C ratio. This value should fall between 0.9-1.1, for samples to be normalized. See example below.

Standardization Gene	Treated/Control Ratio
P1	1.05
S26	1.01
S4	0.87
L12	0.95

Median T/C  
Ratio = 0.98

5. If the median falls outside of this range, adjust the amount of RAGEtag used in the PCR reaction and repeat the normalization procedure. See calculations and explanations on the following page.

6. The following table shows an example where the T/C ratio is outside of the established range (1 uL of 1:250 treated and control sample dilutions were used in PCR). Readjust sample volume for PCR as shown below.

Standardization Gene	Treated/Control Ratio
S11	0.76
L5	0.94
L3	0.82
L27a	0.82
P0	0.69
A52	0.86
L29	1.02

Median T/C  
Ratio = 0.82

- To adjust C sample volume:  $T/C = 0.82$ , or 1.0 T is equivalent to 0.82 C. Therefore, for every 1 uL of T, use 0.82 uL of C.
  - Alternately, adjust volume of T sample:  $T/C = 0.82$  or  $C = T/0.82$ . C is equivalent to  $1/0.82$  T. Use 1.2 uL of T to 1 uL of C. When normalizing multiple test samples, adjust the volume of the test sample while maintaining a constant volume of the control.
7. PCR was rerun using 1 uL of Control sample, and 1.2 uL of Treated sample. The results are shown:

Standardization Gene	Treated/Control Ratio
S11	0.92
L5	0.93
L3	0.85
L27a	0.96
P0	1.02
A52	0.91
L29	1.02

Median T/C  
Ratio = 0.92

- When T/C ratios are recalculated, note that T/C ratios for two genes, L5 and L29, did not change after the first normalization. These genes should be discarded from the analysis. Only genes that respond correctly to the normalization adjustments are considered to be useful as controls for RAGE. Genes with T/C ratios that do not change upon adjustment or shift outside the normalization range are therefore eliminated from statistical analysis.
- Once the samples have been normalized to one another, proceed to Analysis of Specific Genes (Section 5).

## 5 ANALYSIS OF SPECIFIC GENES

To analyze differential expression patterns of specific genes, a combinatorial library of 320 primers is used to selectively amplify any gene or genes of choice using either the A/B or B/A RAGetag as the template. One of the 256 A primers is used in combination with one of the 64 B primers to selectively amplify a subset of the expressed genes. One set of reaction conditions allows the analysis of many genes in one thermocycler run. The RAGE primers are denoted RAGE005 through RAGE332 (See Appendix, Section 8.3).

In a directed analysis of a gene or genes already present in a database, the GS320 search program is used to determine which primers will amplify those specific genes and the orientation of RAGetag that must be isolated to analyze the gene.

The A primers may also be combined pairwise with B primers to scan for novel or unexpected genes that are differentially expressed, using either the A/B or B/A RAGetag as template. The GS320 Database Analysis Software is then used to determine the identity of RAGE amplimers after combinatorial RAGE analysis.

### 5.1 Protocol

#### 5.1.1 PCR Set Up

1. For each sample, set up triplicate PCR reactions for each specific gene to be analyzed. Use the appropriate amount of RAGetag for each sample as determined from the standardization assay above.
2. When performing multiple samples, use of a master mix is recommended. Use the following as a guide for setting up the master mix. (N = the number of samples). An extra reaction volume is included to account for pipettor error.
3. Set up Master Mixes for each sample as shown in the table below. Increase volumes as necessary based on the number of genes being assayed. Set reactions up on ice.

Component	Volume/rxn	Amount in uL (triplicate samples)
<b>RAGetag</b>	Variable	(__ uL x N) + 1 rxn vol. Of RAGetag
<b>5X PCR Buffer</b>	5 uL	(15 uL x N) + 5 uL of 5X PCR Buffer
<b>Taq Pol (1.25 U)</b>	Variable	(__ uL x N) +1.25 U Taq Pol.
<b>Water*</b>	Variable	(__ uL x N) +1 rxn vol of water
<b>Total</b>	<b>23 uL</b>	<b>(69 uL x N) + 23 uL</b>

\*molecular biology grade water

4. For each gene being assayed, aliquot 23 uL of each Master Mix into three 0.2 mL PCR tubes on ice.
5. Add 1 uL of the appropriate RAGE A primer and 1 uL of the appropriate RAGE B primer to each PCR tube on ice. Pulse-spin tubes.
6. Place samples in thermocycler and run using GS320 PCR cycling conditions.

### 5.1.2 GS320 PCR Cycling Conditions:

Condition	Time	Temperature	Cycles
Denaturation	5 minutes	95°C	1 cycle
Denaturation	30 seconds	95°C	28 cycles
Anneal	1 minute	60°C	
Extend	1 minute	72°C	
Final Extension	6 minutes	72°C	1 cycle
Refrigerate	Hold	4°C	

### 5.1.3 Analysis of Results

1. Run 10 uL of each PCR reaction and the appropriate volume of DNA loading dye on an 8% Polyacrylamide gel (16 cm-20 cm plate size or mini gel apparatus).
2. After the run, soak each gel for approximately 30-60 seconds in a 1:10,000 dilution of SYBR Green I, Vistra Green or GelStar diluted in 1X TBE. The same preparation of diluted fluorescent dye may be used to stain multiple gels, but do not store and reuse diluted dye after initial use.
3. Visualize on a Fluorescent Imager.
4. Obtain a densitometric reading for all the bands of interest and average the multiple values for each gene in each sample.
5. Calculate the ratio of T/C (test to control) to determine differences in gene expression between samples.
6. Repeat PCR to confirm expression differences.
7. Verify results (Section 5.2).

## 5.2 Verification of GS320 Results

### 5.2.1 Validation of Products

The GS320 products are usually between 50-400 base pairs in size. Therefore, it is relatively easy to identify the product as the correct gene. There are a number of techniques that may be used to confirm that the GS320 amplicon encodes the expected gene including restriction enzyme digestion, internal PCR and sequencing.

#### A. Size of amplicon

The GS320 Database Analysis Software program determines the size and sequence of the expected product. This can be compared to the size determination of the GS320 amplicon on a polyacrylamide gel. While this internal control is reassuring, it is possible that the band in the gel does not encode the intended gene, as a single pair of GS320 amplicons may generate multiple unique products of the same molecular weight. Therefore, the following assays are also encouraged.

#### B. Validation of Differential Gene Expression

Differential expression of the RAGetag amplicon should be reproducible over a range of concentrations of template. Perform more detailed analysis of initial results by titrating the amount of template used in PCR with all samples. Maintain the standardization ratio of one sample to another during the analysis. The ratio of differential gene expression should remain constant over a linear range of template.

### C. Restriction Enzyme Digestion

The expected amplicon sequence derived from the software may be screened for internal restriction enzyme sites. The GS320 amplicon is digested with the appropriate restriction enzyme and run on a gel. Restriction fragments of the expected size confirm the correct product. Use a nucleic acid analysis program such as Vector NTI Suite (Informax, Rockville, MD) to identify the restriction enzyme sites that will cut the amplicon into at least two distinguishable fragments.

1. Digest 25 uL of the GS320 amplicon reaction with the appropriate restriction enzyme(s).
2. Separate the digested material on the appropriate percentage polyacrylamide gel. Include 25 uL of uncut sample on the gel for comparison.

### D. Gene Specific PCR

Internal gene-specific PCR is also an easy method for GS320 product confirmation. Design gene specific primers using the expected sequence of the GS320 amplicon from the GS320 Database Analysis Software. Use the GS320 amplicon as a template for PCR. The expected size amplicon is confirmed on a polyacrylamide gel.

1. Design a pair of primers for the gene specific fragment using any primer selection software, such as Vector NTI Suite (Informax, Rockville, MD). Determine the predicted size of the gene specific amplicon.
2. Establish conditions for amplification using standard PCR protocols. Use 1 uL of a 1:500-1:2000 dilution of the GS320 PCR amplicon in the reaction depending on the amount of amplicon obtained from the initial GS320 amplification.
3. Size the amplified material on the appropriate percentage polyacrylamide gel.

### E. Sequencing

GS320 PCR products may be identified by DNA sequence analysis. If multiple products are generated from a single pair of RAGE primers it may first be necessary to purify the band of interest. Use standard purification methods, and scale up reactions to generate additional product as necessary. RAGE primers can be used as sequencing primers.

#### 5.2.2 Validation of Expression Profiles

Expression profiles identified with GS320 can be confirmed by multiple methods.

1. RNase Protection Assay
2. Northern Blotting
3. RT-PCR
4. Microarray

## 6 ADDITIONAL PROTOCOLS

### 6.1 Preparation of Total RNA or Poly(A) mRNA

- Either total RNA or Poly(A) mRNA can be used for GS320. The RNA must be of high quality in order to ensure full-length cDNA synthesis. We recommend the following kits:

**Total RNA isolation:**

- **Ambion:** RNAwiz (Cat #9736) or RNAaqueous-4PCR (Cat #1914)
- **Invitrogen:** Trizol (Cat #15596)

**For Poly(A) mRNA isolation:**

- **Ambion:** Poly(A)Pure (Cat #1915)
- **Invitrogen:** FASTTRACK 2.0 (Cat #K1593-02).

- Practice the appropriate procedures for handling and storing RNA, using RNase-free reagents, tubes and tips. Gloves should always be worn when working with RNA.
- The quality and purity of the RNA may be assessed by measuring the A260:A280 ratio, which should be between 1.7 and 2.1. If the ratio is lower than 1.7 it is indicative of protein, phenol, or other contamination. Contaminants can often be removed by further purification. To remove proteins perform a phenol-chloroform extraction and ethanol precipitation. To remove phenol use chloroform or chloroform:isoamyl alcohol extraction and ethanol precipitation. Other recommendations may be found in the manuals of the specific RNA Isolation kit.
- RNA must be free of genomic DNA contamination. DNase treat RNA as necessary. We recommend Ambion's DNA-free™ Kit (Cat. No. 1906).
- Visualize the RNA by denaturing agarose gel electrophoresis.
  - **Total RNA:** Run 0.5 ug of RNA on a 1% formaldehyde gel. Stain with SYBR Green II or with ethidium bromide using standard protocols. The 28S and 18S ribosomal RNA should appear as sharp, discrete bands.
  - **Poly(A) mRNA:** Run 200–500 ng on a 1% formaldehyde gel. Stain with SYBR Green II. The RNA should appear as a smear ranging from 200 bp to greater than 10 kb.

### 6.2 cDNA Synthesis

Material for cDNA synthesis can be purchased as a complete kit or individual components can be obtained separately from a variety of vendors. When appropriate, we have recommended vendors that work well with our system. Recipes for cDNA synthesis buffers and recommended vendor information for individual components may be found in Section 8.1 and 8.2. Use the following procedure with your choice of enzyme listed below. If using individual reagents follow the guidelines below for final concentrations of components and adjust the volume of the reactions accordingly.

When using a method for cDNA synthesis not listed above substitute 2 ug of Biotinylated Oligo(dT) (6.5 uL of stock solution provided in the RAGETag Synthesis Kit) for the primer in first strand synthesis. This quantity of primer is sufficient for use with 2-5 ug of mRNA or 10-20 ug of total RNA. Adjust final first strand reaction volumes accordingly. Proceed with the rest of protocol as suggested by vendor. After synthesis of biotinylated cDNA proceed to generation of A/B and/or B/A RAGETags (Sections 3.1.2 and 3.1.3)

## 6.2.1 Materials and Equipment Needed for cDNA Synthesis

- 10-20 ug total RNA or 2-5 ug Poly(A) mRNA
- **cDNA Synthesis Kits**
  - cDNA Synthesis Kit (Invitrogen Cat. No. 18267-013)
  - SuperScript Choice System (Invitrogen Cat. No. 18090)

**Or:**

- **Individual Components for cDNA Synthesis (Recipes in Section 8.1)**
- Reverse Transcriptase:
  - M-MLV (Invitrogen Cat. No. 28025-013)
  - SuperScript RT (Invitrogen Cat. No. 18053-017)
  - SuperScript II RT (Invitrogen Cat. No. 18064-022, 18064-014)
- 5X 2<sup>nd</sup> Strand Buffer (Invitrogen Cat. No. 18012-014)
- 10 mM dNTP Mix (Invitrogen Cat. No. 18427-013) (MBI Fermentas Cat. No. R0191, R01920)
- 0.1 M DTT (Invitrogen Cat. No. 15508-013)
- *E. coli* RNase H (Invitrogen Cat. No. 18021-014, 18021-071)
- *E. coli* DNA Polymerase I (Invitrogen Cat. No. 18010-017, 18010-025)
- *E. coli* DNA Ligase (Invitrogen Cat. No. 18052-019)
- Biotinylated oligo(dT) from RAGEtag Synthesis Kit (800-0001)
- RNase A/T1 (Ambion Cat. No. 2286) **Only** for synthesis from Total RNA
- DEPC-treated water
- Glycogen (20 mg/mL) from RAGEtag Synthesis Kit (800-0014)
- 10 M Ammonium Acetate
- 100% Ethanol
- 70% (v/v) ethanol
- DNA-free<sup>TM</sup> (Ambion Cat. No. 1906)
- Sterile 1.5 mL and 0.65 mL Low Adhesion microfuge tubes (KPL Cat No. 60-00-54, 60-00-55)
- Disposable gloves
- Microcentrifuge
- Water Baths, Heat Blocks or Thermocyclers at 14°C, 16°C, 37°C, 42°C, and 70°C

## 6.2.2 cDNA Synthesis Protocol

Either poly(A) mRNA (2- 5 ug) or total RNA (10-20 ug) may be used as starting material for this kit. Total RNA must first be DNase treated to eliminate genomic DNA contamination. We routinely use DNA-free™ (Ambion, Catalog # 1906).

### A. First Strand Synthesis:

1. Components for 1<sup>st</sup> strand synthesis
  - a) 5X First Strand Buffer
  - b) Biotinylated Oligo(dT)
  - c) 0.1 M DTT
  - d) 10 mM dNTP
  - e) M-MLV or SuperScript or SuperScript II Enzyme
  - f) DEPC-treated Water
2. Set up the following on ice:

<u>Component</u>	<u>Volume</u>
mRNA (2-5 ug)	Variable
or	
Total RNA (10-20 ug)	
10 mM dNTP Mix	2.5 uL
Biotinylated Oligo(dT) (provided in RAGETag Synthesis Kit)	6.5 uL
<u>DEPC-treated Water</u>	<u>Variable</u>
<b>Final Volume</b>	<b>32.5 uL</b>

3. Incubate at 70<sup>0</sup>C for 5 minutes. and chill on ice.
4. Add the following to tube on ice:

<u>Component</u>	<u>Volume</u>
5X First Strand Buffer	10 uL
0.1 M DTT	5 uL
<u>Reverse Transcriptase (500 units)</u>	<u>variable</u>
<b>Final Volume</b>	<b>17.5 uL</b>

5. Mix contents of tube gently.
6. Incubate for 1 hour at 37<sup>0</sup>C.

### B. Second Strand Synthesis:

1. Components for 2<sup>nd</sup> Strand Synthesis:
  - a) 10X or 5X 2<sup>nd</sup> Strand Buffer
  - b) *E. coli* DNA Polymerase I
  - c) *E. coli* RNase H
  - d) *E. coli* DNA Ligase
  - e) 10 mM dNTP
  - f) DEPC-treated Water

2. Place tube from First Strand Synthesis on ice and add the following:

<u>Component</u>	<u>Volume</u>
DEPC-treated Water	289.5 uL
10 mM dNTP Mix	7.5 uL
10X Second Strand Buffer (if using 5X 2 <sup>nd</sup> Strand buffer, adjust volumes accordingly)	40 uL
DNA Polymerase I (100 units)	10 uL
RNase H (3.5 units)	1.75 uL
DNA Ligase (12.5 units)	1.25 uL

3. Mix contents of tube(s).
4. Incubate for 2-4 hours at 16°C.
5. RNase Step:
- Poly(A) mRNA:** Proceed to processing step C. Samples may be stored at -20°C until processing.
  - Total RNA:** cDNA made from total RNA must be RNase treated as follows:
    - Add 5 uL of RNase A/T1 (Ambion, Cat. No. 2286).
    - Incubate at 37°C for 30 minutes.
    - Proceed to Processing Step C. Procedure may be stopped at this point and sample may be stored at -20°C.

### C. Processing Step:

- Place 700 uL of cold 100% ethanol into a low adhesion 1.5 mL microcentrifuge tube.
- Flick tube of KPL's PureBind Beads until the beads are in suspension.
- Pipette 70 uL of beads into the tube with ethanol. Mix well by inversion. Pulse-spin 1-2 seconds and place tube into magnet.
- Remove supernatant. **Immediately remove tube from magnet and place in holder on benchtop.** Add 700 uL of 100 % cold ethanol. Mix by inverting the tube until a suspension is formed. Pulse-spin 1-2 seconds.
- Return tube to magnet and remove supernatant. Repeat wash 1X as above.
- After last wash, resuspend beads in 700 uL 100 % ethanol and place on ice.
- To the 400 uL of cDNA sample, add 6 uL glycogen and 200 uL 10M Ammonium Acetate. Mix well by inversion. Pulse-spin 1-2 seconds.
- Add DNA solution to beads and mix by inverting the tube. Pulse-spin 1-2 seconds.
- Place on ice for 15 minutes.
- Bind to magnet and remove supernatant.
- Wash three times with 700 uL of cold 70% ethanol. Don't pipette the beads in the ethanol - mix by inverting tube until a suspension is formed. Pulse-spin 1-2 seconds prior to placing tube in magnet.
- On last wash, remove as much 70% ethanol as possible. Remove tube from magnet and pulse-spin 1-2 seconds in a microcentrifuge. Return tube to magnet and remove residual 70% ethanol.
- Dry beads 3 minutes at room temperature. Monitor drying carefully-**Do not** let the bead pellet crack or cake in the tube.
- Resuspend beads in 25 uL of 1X TE. Vortex. Let sit at room temperature 10 minutes. Vortex briefly and pulse-spin 1-2 seconds in microcentrifuge. Bind to magnet and **keep** supernatant with cDNA sample. Discard beads.

15. Store cDNA sample at  $-20^{\circ}\text{C}$  or quantitate as described below.

### 6.2.3 cDNA Quantitation

1. Make a 1:50 dilution of sample in 1X TE (i.e. 2  $\mu\text{L}$  of cDNA to 98  $\mu\text{L}$  1X TE) in a low adhesion microcentrifuge tube. Mix well by vortexing.
2. Read  $\text{OD}_{260}$  on a spectrophotometer.
  - Use matching quartz cuvetts or a 96-well UV-spectrophotometric plate. In order to use minimal amounts of cDNA for quantitation, we recommend use of ultra-micro cuvetts that hold a minimum volume of 50  $\mu\text{L}$ .
  - Use 1X TE as the blanking solution.
  - The  $\text{OD}_{260}$  reading should fall between 0.1 and 1.0. If necessary, requantitate using a higher or lower dilution of cDNA.
  - $\text{OD}_{260}$  of 1 = 50 ng/ $\mu\text{L}$  cDNA.
3. Calculate cDNA concentration using the following formula:

$$\text{OD}_{260} \times \text{dilution factor} \times 50 = \text{cDNA concentration (ng/\mu L)}$$

### 6.2.4 cDNA Quality Check

- Use KPL's cDNA Integrity Kit (Catalog #60-06-00) to ensure that cDNA is full-length and of quality that is amplifiable in PCR.
- Additionally, run approximately 200 ng of cDNA sample on a 1% agarose Ethidium Bromide gel. Full-length material should appear as a smear ranging from approximately 5 kb to 500 bp. A 2-4 kb prominent band may be evident in some species.

### 6.3 Synthesis of biotinylated probes from GS320 Amplimers

It is possible to synthesize biotinylated DNA or RNA probes directly from the GS320 amplimer. These may be used as nucleic acid probes for Northern blotting, Southern blotting or for screening a library.

#### A. DNA Probes:

The average size of the GS320 amplimer is ~125 bp, which makes it ideal for using as a template in random priming. Biotinylated probes may be generated from GS320 amplimers using random primer biotinylation (KPL Cat. No. 60-01-00). If multiple products are present in the sample to be biotinylated it may first be necessary to purify the band of interest.

#### B. RNA Probes:

RNA probes can be generated from GS320 amplimers. The technique utilizes a primer pair that adds the T7 and SP6 GS320 promoter sequences to the ends of the GS320 amplimer during PCR. The modified GS320 amplimer is then biotinylated by *in vitro* transcription.

1. Amplify the desired GS320 amplimer with the GS320 Universal T7/SP6 primer set (KPL Cat. No. 800-0073).
2. Biotinylate either strand of RNA using KPL's RNA *in vitro* transcription Biotinylation Kit (Cat. No. 60-01-02).

#### A/B Orientation Amplimers

1. antisense probe: Use SP6 Polymerase
2. sense probe: Use T7 Polymerase

#### B/A Orientation Amplimers

1. antisense probe: Use T7 Polymerase
2. sense probe: Use SP6 Polymerase

## 7 TROUBLESHOOTING

### 7.1 cDNA Synthesis

Problem	Possible Cause	Corrective Measure
No full-length cDNA	<ul style="list-style-type: none"> <li>degraded RNA</li> <li>cDNA not full length due to poor first or second strand cDNA synthesis</li> </ul>	<ul style="list-style-type: none"> <li>Use careful handling procedures during isolation of RNA.</li> <li>Make sure cDNA synthesis reagents are of high quality and have not expired.</li> <li>Maintain proper storage of all reagents for cDNA synthesis.</li> <li>Run appropriate controls during cDNA synthesis.</li> <li>Use KPL's cDNA Integrity Kit to check for full-length cDNA.</li> </ul>
Low yield of cDNA	<ul style="list-style-type: none"> <li>Sub-optimal Reverse Transcriptase</li> </ul>	<ul style="list-style-type: none"> <li>Use recommended Reverse Transcriptases (Sections 6.2.1 and 8.2)</li> </ul>

### 7.2 Determination of Linear Range of PCR

Problem	Possible Cause	Corrective Measure
<ul style="list-style-type: none"> <li>No amplicon</li> </ul>	<ul style="list-style-type: none"> <li>Not enough RAGEtag made.</li> <li>cDNA not quantitated correctly.</li> <li>cDNA not full length due to poor first or second strand synthesis.</li> <li>Wrong RAGEtag orientation used for PCR amplification.</li> </ul>	<ul style="list-style-type: none"> <li>Perform all steps of RAGEtag Synthesis carefully to maintain high yields at each step.</li> <li>Requantitate cDNA. Start RAGEtag synthesis with 1-2 ug cDNA.</li> <li>Make sure cDNA synthesis reagents are of high quality and have not expired.</li> <li>Maintain proper storage of all reagents for cDNA synthesis.</li> <li>Run appropriate controls during cDNA synthesis.</li> <li>Use cDNA Integrity Kit to check for full-length cDNA.</li> <li>Use correct RAGEtag orientation to amplify gene of interest.</li> </ul>

## Troubleshooting: Determination of Linear Range of PCR, (continued)

<b>Problem</b>	<b>Possible Cause</b>	<b>Corrective Measure</b>
<ul style="list-style-type: none"> <li>Difficulty finding linear range of reaction with standardization genes.</li> </ul>	<ul style="list-style-type: none"> <li>Fluorescent imaging parameters incorrect.</li> <li>Not enough RAGETag used in PCR.</li> <li>Did not analyze enough standardization genes.</li> </ul>	<ul style="list-style-type: none"> <li>Configure instrument correctly.</li> <li>Use fresh SYBR Green.</li> <li>Use more or less RAGETag as appropriate. Use at least 5 different dilutions of the RAGETag in PCR.</li> <li>Titrate the dilutions of RAGETag as necessary to find the linear range of the reaction using at least 5 genes.</li> <li>Standardization genes that fall out of the linear range compared to the majority of the rest of the genes should not be used for the linear range calculation.</li> </ul>

### 7.3 Standardization

Standardization genes do not adjust as expected with normalization.	Expression of the particular gene is affected by treatment in your system.	Do not use results from genes that do not adjust with changes in RAGETag concentration for normalization analysis.
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### 7.4 Detection of Specific Genes

<b>Problem</b>	<b>Possible Cause</b>	<b>Corrective Measure</b>
Expected amplification product not observed.	<ul style="list-style-type: none"> <li>Gene of interest is not expressed in your system.</li> <li>Not enough RAGETag used as template.</li> <li>Component left out of PCR reaction.</li> <li>PCR conditions not optimal.</li> </ul>	<ul style="list-style-type: none"> <li>Increase the amount of RAGETag used as template for PCR to confirm your results.</li> <li>Repeat PCR using more RAGETag. A titration of RAGETag may be necessary.</li> <li>Repeat PCR using a master mix to minimize errors in pipetting.</li> <li>Determine optimal annealing temperature using an annealing temperature gradient during PCR (55°C to 70°C).</li> </ul>

## Troubleshooting: Detection of Specific Genes (continued)

Problem	Possible Cause	Corrective Measure
Expected amplification product not observed (continued).	<ul style="list-style-type: none"> <li>• Wrong primer pair used.</li> <li>• Wrong RAGETag orientation used for PCR amplification.</li> <li>• Fluorescent imaging parameters incorrect.</li> <li>• Agarose gel used.</li> <li>• Incompatible Taq polymerase.</li> <li>• cDNA does not contain biotinylated 3' end.</li> </ul>	<ul style="list-style-type: none"> <li>• Ensure that the correct primer pair is used for the gene of interest and the search is performed using the correct species.</li> <li>• Use correct RAGETag orientation to amplify gene of interest.</li> <li>• Use fresh SYBR Green.</li> <li>• Set up instrument correctly.</li> <li>• Use polyacrylamide gels and SYBR Green to visualize PCR products.</li> <li>• Follow suggestions as to which Taq polymerase to use (Section 8/2).</li> <li>• Prepare cDNA using biotinylated oligo(dT).</li> </ul>
Spurious or laddering banding pattern seen on gel.	<ul style="list-style-type: none"> <li>• Non-specific amplification due to too much template.</li> <li>• PCR conditions not optimal.</li> </ul>	<ul style="list-style-type: none"> <li>• Decrease the amount of RAGETag used in the PCR reaction.</li> <li>• Determine optimal annealing temperature using an annealing temperature gradient during PCR. (55°C to 70°C).</li> <li>• Titrate MgCl<sub>2</sub> concentrations in the PCR reactions.</li> <li>• Use DMSO or betaine in the PCR reaction to enhance specificity.</li> </ul>
Multiple PCR products.	Additional amplimers may occur due to amplification of multiple genes by a single primer pair.	<ul style="list-style-type: none"> <li>• Search the GS320 Database Analysis Software for other genes that may be amplified by the primer pair.</li> <li>• If the gene is not found in the database, sequence it to determine if it is a novel gene.</li> </ul>

## 8 APPENDIX

### 8.1 Reagent Preparation

#### **10M Ammonium Acetate**

Dissolve 385.4 g ammonium acetate in 150 mL of molecular biology grade water. QS water to 500 mL. Filter sterilize and store at room temperature.

#### **10 mM dNTP Mix**

10 mM dATP

10 mM dCTP

10 mM dGTP

10 mM dTTP

#### **10X DNA Sample Loading Buffer**

20% Ficoll

0.25% Bromophenol Blue

0.1% SDS

**or:**

#### **10X Urea DNA Sample Loading Buffer**

7.0 M Urea

0.4 % Bromophenol Blue

50 mM Tris, pH 8.0

20 mM EDTA, pH 8.0

#### **TE Buffer**

10 mM Tris, pH 8.0

1 mM EDTA, pH 8.0

#### **5X 1<sup>st</sup> Strand cDNA Synthesis Buffer**

(for use with M-MLV, Superscript and SuperScript II)

250 mM Tris-HCl, pH 8.3

375 mM KCl

15 mM MgCl<sub>2</sub>

#### **5X 2nd Strand cDNA Synthesis Buffer**

(for use with M-MLV, Superscript and SuperScript II)

100 mM Tris-HCl, pH 6.9

23 mM MgCl<sub>2</sub>

450 mM KCl

50 mM (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub>

0.75 mM B-NAD<sup>+</sup>

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### **TE-saturated Phenol:Chloroform:Isoamyl Alcohol**

Mix equal parts of TE buffer and phenol and allow the phase to separate. Then mix 1 part of the lower phenol phase with 1 part of chloroform:isoamyl alcohol (24:1).

## **8.2 Suggested Sources for Supplies and Equipment**

### **cDNA Integrity Kit**

- KPL (Cat No. 60-06-00)

### **cDNA Synthesis Components**

- 0.1 M DTT (Invitrogen Cat. No. 15508-013)
- M-MLV Reverse Transcriptase (Invitrogen Cat. No. 28025-013)
- SuperScript II Reverse Transcriptase (Invitrogen Cat. No. 18064-014)
- SuperScript Reverse Transcriptase (Invitrogen Cat. No. 18053-017)
- *E. coli* RNase H (Invitrogen Cat. No. 18021-014, 18021-071)
- *E. coli* DNA Polymerase I (Invitrogen Cat. No. 18010-017, 10-025)
- *E. coli* DNA Ligase (Invitrogen Cat. No. 18052-019)
- RNase A/TI (Ambion Cat. No. 2286)

### **cDNA Synthesis Kits**

- Invitrogen: cDNA Synthesis Kit (Cat. No. 18267-013) or Superscript Choice System (Cat. No. 18090-019).

### **DNase Treatment**

- DNA-free™ (Ambion, Catalog # 1906).

### **Fluorescent Imager**

- Syngene. GeneGenius Tel: 1-877-435-3627.

### **Fluorescent Nucleic Acid Stains**

- **DNA**
  - BioWhittaker: SYBR Green I (Cat. No. 50513) or GelStar (Cat. No. 50535)
  - Amersham/Pharmacia: Vistra Green (Cat. No. RPN5786).
  - Ethidium Bromide (variety of sources)
- **RNA**
  - BioWhittaker: SYBR Green II (Cat. No. 50523)

### **Low Adhesion Microcentrifuge Tubes**

- KPL: 1.7 mL (250/box) (Cat. No. 60-00-55) and 0.65 mL (500/box) (Cat. No. 60-00-54)

### **Magnetic Particle Concentrator**

- KPL (Cat. No. 60-00-52)

### **Non-Isotopic Hybridization and Detection Kits**

- KPL RNADetector Northern Blotting Kits
  - 500 cm<sup>2</sup> (Cat. No. 54-30-02)
  - 2000 cm<sup>2</sup> (Cat. No. 54-30-01)
- KPL DNADetector Southern Blotting Kit
  - Genomic Southern Blotting Kit (Cat. No. 54-30-03)

- HRP Southern Blotting Kit (Cat. No. 54-30-00)

### **Nucleic Acid Biotin Probe Labeling**

- **DNA**
  - KPL Detector PCR DNA Biotinylation Kit (Cat. No. 60-01-01)
  - KPL Detector Random Primer DNA Biotinylation Kit (Cat. No. 60-01-00)
- **RNA**
  - KPL Detector RNA in vitro Transcription Biotinylation Kit (Cat. No. 60-01-02)

### **Poly (A) mRNA Isolation**

- Invitrogen: Fasttrack 2.0 (Cat. No. K1593-02)
- Ambion: Poly(A)Pure™ Isolation Kit (Cat. No. 1915)

### **Polyacrylamide Gels (Pre-cast)**

- Invitrogen/Novex

### **RNase Treatment**

- RNase A/T1 (Ambion, Cat. No. 2286)

### **Taq Polymerase**

- MBI Fermentas Taq
  - Native (Cat. No. EPO281)
  - Recombinant (Cat. No. EPO401)
- Invitrogen Taq
  - Native (Cat. No. 18038-018)
  - Recombinant (Cat. No. 10342-053)
  - Platinum Taq (Cat. No. 10966-018)
- Promega Taq Beads (Cat. No. M5661)
- PE Amplitaq (Cat. No. N808-0161)

### **Total RNA Purification**

- Ambion: RNAwiz (Cat. No. 9736) or RNAaqueous-4PCR (Cat No. 1914)
- Invitrogen: TRIzol® Reagent (Cat. No. 15596)

### **Universal T7/SP6 Primer Set**

- KPL: GS320 Universal T7/SP6 Primer Set (25 reactions) (Cat. No. 800-0073)

## 8.3 RAGE Primers

### 8.3.1 A PRIMERS

Oligo	Catalog Number	Oligo	Catalog Number	Oligo	Catalog Number
RAGE 005	800-1005	RAGE 049	800-1049	RAGE 092	800-1092
RAGE 006	800-1006	RAGE 050	800-1050	RAGE 093	800-1093
RAGE 007	800-1007	RAGE 051	800-1051	RAGE 094	800-1094
RAGE 008	800-1008	RAGE 052	800-1052	RAGE 095	800-1095
RAGE 009	800-1009	RAGE 053	800-1053	RAGE 096	800-1096
RAGE 010	800-1010	RAGE 054	800-1054	RAGE 097	800-1097
RAGE 011	800-1011	RAGE 055	800-1055	RAGE 098	800-1098
RAGE 012	800-1012	RAGE 056	800-1056	RAGE 099	800-1099
RAGE 013	800-1013	RAGE 057	800-1057	RAGE 100	800-1100
RAGE 014	800-1014	RAGE 058	800-1058	RAGE 101	800-1101
RAGE 015	800-1015	RAGE 059	800-1059	RAGE 102	800-1102
RAGE 016	800-1016	RAGE 060	800-1060	RAGE 103	800-1103
RAGE 017	800-1017	RAGE 061	800-1061	RAGE 104	800-1104
RAGE 018	800-1018	RAGE 062	800-1062	RAGE 105	800-1105
RAGE 019	800-1019	RAGE 063	800-1063	RAGE 106	800-1106
RAGE 020	800-1020	RAGE 064	800-1064	RAGE 107	800-1107
RAGE 028	800-1028	RAGE 065	800-1065	RAGE 108	800-1108
RAGE 029	800-1029	RAGE 066	800-1066	RAGE 109	800-1109
RAGE 030	800-1030	RAGE 077	800-1077	RAGE 110	800-1110
RAGE 031	800-1031	RAGE 078	800-1078	RAGE 111	800-1111
RAGE 032	800-1032	RAGE 079	800-1079	RAGE 112	800-1112
RAGE 033	800-1033	RAGE 080	800-1080	RAGE 113	800-1113
RAGE 034	800-1034	RAGE 081	800-1081	RAGE 114	800-1114
RAGE 035	800-1035	RAGE 082	800-1082	RAGE 115	800-1115
RAGE 036	800-1036	RAGE 083	800-1083	RAGE 116	800-1116
RAGE 037	800-1037	RAGE 084	800-1084	RAGE 117	800-1117
RAGE 038	800-1038	RAGE 085	800-1085	RAGE 118	800-1118
RAGE 039	800-1039	RAGE 086	800-1086	RAGE 119	800-1119
RAGE 040	800-1040	RAGE 087	800-1087	RAGE 120	800-1120
RAGE 041	800-1041	RAGE 088	800-1088	RAGE 121	800-1121
RAGE 042	800-1042	RAGE 089	800-1089	RAGE 122	800-1122
RAGE 043	800-1043	RAGE 090	800-1090	RAGE 123	800-1123
RAGE 048	800-1048	RAGE 091	800-1091	RAGE 124	800-1124

**A Primers (continued)**

<b>Oligo</b>	<b>Catalog Number</b>	<b>Oligo</b>	<b>Catalog Number</b>	<b>Oligo</b>	<b>Catalog Number</b>
RAGE 125	800-1125	RAGE 160	800-1160	RAGE 194	800-1194
RAGE 126	800-1126	RAGE 161	800-1161	RAGE 195	800-1195
RAGE 127	800-1127	RAGE 162	800-1162	RAGE 196	800-1196
RAGE 128	800-1128	RAGE 163	800-1163	RAGE 197	800-1197
RAGE 129	800-1129	RAGE 164	800-1164	RAGE 198	800-1198
RAGE 130	800-1130	RAGE 165	800-1165	RAGE 199	800-1199
RAGE 131	800-1131	RAGE 166	800-1166	RAGE 200	800-1200
RAGE 132	800-1132	RAGE 167	800-1167	RAGE 201	800-1201
RAGE 133	800-1133	RAGE 168	800-1168	RAGE 202	800-1202
RAGE 134	800-1134	RAGE 169	800-1169	RAGE 203	800-1203
RAGE 135	800-1135	RAGE 170	800-1170	RAGE 204	800-1204
RAGE 136	800-1136	RAGE 171	800-1171	RAGE 205	800-1205
RAGE 137	800-1137	RAGE 172	800-1172	RAGE 206	800-1206
RAGE 138	800-1138	RAGE 173	800-1173	RAGE 207	800-1207
RAGE 139	800-1139	RAGE 174	800-1174	RAGE 208	800-1208
RAGE 140	800-1140	RAGE 175	800-1175	RAGE 209	800-1209
RAGE 141	800-1141	RAGE 176	800-1176	RAGE 210	800-1210
RAGE 142	800-1142	RAGE 177	800-1177	RAGE 211	800-1211
RAGE 143	800-1143	RAGE 178	800-1178	RAGE 212	800-1212
RAGE 144	800-1144	RAGE 179	800-1179	RAGE 213	800-1213
RAGE 145	800-1145	RAGE 180	800-1180	RAGE 214	800-1214
RAGE 146	800-1146	RAGE 181	800-1181	RAGE 215	800-1215
RAGE 147	800-1147	RAGE 182	800-1182	RAGE 216	800-1216
RAGE 148	800-1148	RAGE 183	800-1183	RAGE 217	800-1217
RAGE 149	800-1149	RAGE 184	800-1184	RAGE 218	800-1218
RAGE 150	800-1150	RAGE 185	800-1185	RAGE 219	800-1219
RAGE 151	800-1151	RAGE 186	800-1186	RAGE 220	800-1220
RAGE 152	800-1152	RAGE 187	800-1187	RAGE 221	800-1221
RAGE 153	800-1153	RAGE 188	800-1188	RAGE 222	800-1222
RAGE 154	800-1154	RAGE 189	800-1189	RAGE 223	800-1223
RAGE 155	800-1155	RAGE 190	800-1190	RAGE 224	800-1224
RAGE 156	800-1156	RAGE 191	800-1191	RAGE 225	800-1225
RAGE 157	800-1157	RAGE 192	800-1192	RAGE 226	800-1226
RAGE 158	800-1158	RAGE 193	800-1193	RAGE 227	800-1227
RAGE 159	800-1159			RAGE 228	800-1228

**A Primers** (continued)

<b>Oligo</b>	<b>Catalog Number</b>	<b>Oligo</b>	<b>Catalog Number</b>	<b>Oligo</b>	<b>Catalog Number</b>
RAGE 229	800-1229	RAGE 246	800-1246	RAGE 264	800-1264
RAGE 230	800-1230	RAGE 247	800-1247	RAGE 265	800-1265
RAGE 231	800-1231	RAGE 248	800-1248	RAGE 266	800-1266
RAGE 232	800-1232	RAGE 249	800-1249	RAGE 267	800-1267
RAGE 233	800-1233	RAGE 250	800-1250	RAGE 268	800-1268
RAGE 234	800-1234	RAGE 251	800-1251	RAGE 269	800-1269
RAGE 235	800-1235	RAGE 252	800-1252	RAGE 270	800-1270
RAGE 236	800-1236	RAGE 253	800-1253	RAGE 271	800-1271
RAGE 237	800-1237	RAGE 254	800-1254	RAGE 272	800-1272
RAGE 238	800-1238	RAGE 255	800-1255	RAGE 273	800-1273
RAGE 239	800-1239	RAGE 256	800-1256	RAGE 274	800-1274
RAGE 240	800-1240	RAGE 257	800-1257	RAGE 275	800-1275
RAGE 241	800-1241	RAGE 258	800-1258	RAGE 276	800-1276
RAGE 242	800-1242	RAGE 259	800-1259	RAGE 277	800-1277
RAGE 243	800-1243	RAGE 260	800-1260	RAGE 278	800-1278
RAGE 244	800-1244	RAGE 261	800-1261	RAGE 279	800-1279
RAGE 245	800-1245	RAGE 262	800-1262	RAGE 280	800-1280
		RAGE 263	800-1263	RAGE 281	800-1281

**8.3.2 B PRIMERS**

<b>Oligo</b>	<b>Catalog Number</b>	<b>Oligo</b>	<b>Catalog Number</b>	<b>Oligo</b>	<b>Catalog Number</b>
RAGE 021	800-1021	RAGE 291	800-1291	RAGE 313	800-1313
RAGE 022	800-1022	RAGE 292	800-1292	RAGE 314	800-1314
RAGE 023	800-1023	RAGE 293	800-1293	RAGE 315	800-1315
RAGE 024	800-1024	RAGE 294	800-1294	RAGE 316	800-1316
RAGE 044	800-1044	RAGE 295	800-1295	RAGE 317	800-1317
RAGE 045	800-1045	RAGE 296	800-1296	RAGE 318	800-1318
RAGE 046	800-1046	RAGE 297	800-1297	RAGE 319	800-1319
RAGE 047	800-1047	RAGE 298	800-1298	RAGE 320	800-1320
RAGE 067	800-1067	RAGE 299	800-1299	RAGE 321	800-1321
RAGE 068	800-1068	RAGE 300	800-1300	RAGE 322	800-1322
RAGE 069	800-1069	RAGE 301	800-1301	RAGE 323	800-1323
RAGE 070	800-1070	RAGE 302	800-1302	RAGE 324	800-1324
RAGE 071	800-1071	RAGE 303	800-1303	RAGE 325	800-1325
RAGE 282	800-1282	RAGE 304	800-1304	RAGE 326	800-1326
RAGE 283	800-1283	RAGE 305	800-1305	RAGE 327	800-1327
RAGE 284	800-1284	RAGE 306	800-1306	RAGE 328	800-1328
RAGE 285	800-1285	RAGE 307	800-1307	RAGE 329	800-1329
RAGE 286	800-1286	RAGE 308	800-1308	RAGE 330	800-1330
RAGE 287	800-1287	RAGE 309	800-1309	RAGE 331	800-1331
RAGE 288	800-1288	RAGE 310	800-1310	RAGE 332	800-1332
RAGE 289	800-1289	RAGE 311	800-1311		
RAGE 290	800-1290	RAGE 312	800-1312		

**9 MATERIAL SAFETY DATA SHEET**

REVIEW DATE 11/13/00

**1. IDENTIFICATION**

*Hsp92II*  
*DpnII*  
 T4 DNA Ligase

**Catalog No:**

800-0006  
 800-0009  
 800-0011

**2. COMPOSITION AND INGREDIENTS INFORMATION**

<u>Reagent</u>	<u>Weight %</u>	<u>CAS #</u>
Glycerol	50%	56-81-5

**3. HAZARDS IDENTIFICATION**

Skin and eye irritant

**4. FIRST-AID PROCEDURES**Inhalation: Remove subject to fresh air. Seek medical attention if necessary.Eye and Skin Contact: Flush eyes or skin with copious amounts of water. Wash contaminated clothing before reuse. Consult a physician if irritation persists.**5. FIRE FIGHTING PROCEDURES**Extinguishing Media: Foam, Alcohol Foam, CO<sub>2</sub>, Dry Chemical and Water/FogUnusual Fire and Explosion Hazards: Upon evaporation of water, glycerol may emit toxic fumes under fire conditions. Wear self-contained breathing apparatus and protective clothing to prevent contact with skin and eyes.**6. ACCIDENTAL RELEASE MEASURES**

Contain spill and clean-up with copious amounts of water. Avoid contact with skin and clothing.

**7. EXPOSURE CONTROL (PERSONAL PROTECTIVE EQUIPMENT)**Inhalation: Adverse health effects from vapors and spray mist in poorly ventilated areas may include irritation of the mucous membranes of the nose, throat, respiratory tract and symptoms of headache and nausea.Eye Contact: Direct contact with product may result in eye irritation.Skin Contact: Prolonged and repeated contact with product may cause skin irritation.Respiratory Protection: None required if good ventilation is maintained. Otherwise wear MSHA/NIOSH approved respirator suitable for vapor or mist concentrations encountered.**8. HANDLING AND STORAGE**

Store at -20°C

**9. PHYSICAL AND CHEMICAL PROPERTIES**Appearance: Clear, colorless solutionMelting or Freezing Point: <0°C/32°F (water)Boiling Point: 100°C/212°F (water)Solubility in Water: Dilutable**10. STABILITY AND REACTIVITY INFORMATION**

Stable

**11. TOXICOLOGICAL INFORMATION**Hazardous Decomposition Products: Carbon Monoxide, Carbon DioxideHazardous Polymerization: Will not occurIncompatibility Materials to Avoid: Strong oxidizing agents and strong bases**12. ECOLOGICAL INFORMATION**

N/A

**13. DISPOSAL GUIDELINES**

Observe all Federal, State and Local laws concerning health and pollution.

**14. TRANSPORT INFORMATION**

N/A

**15. REGULATORY INFORMATION AND CONSIDERATIONS**

This product is a mixture that may contain one or more hazardous chemicals. The hazardous ingredients listed are only those as required by 29 CFR 1910.1200 g 2.C1

**16. OTHER**

The above information is believed to be correct, but does not purport to be all inclusive and shall be used only as a guide. KPL shall not be held liable for any damage resulting from handling or from

contact with the above product. Users should make their own investigations to determine the suitability of the information for their particular purposes.