QuantumRNA™ ß-actin Internal Standards

Part Number AM1720

QuantumRNA™ ß-actin Internal Standards Kit

(Part Number AM1720)

Protocol

P/N 1720M Revision C **Revision Date: September 7, 2010**

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I. Introduction

A. Introduction to Relative Quantitative RT-PCR

Reverse Transcription (RT), coupled with the Polymerase Chain Reaction (PCR) has literally revolutionized the study of gene expression. It is now possible to characterize the expression profile of any gene, regardless of the amount of starting material or the abundance of the mRNA transcript. In RT-PCR, an RNA template is copied into a complementary DNA transcript (a cDNA) using a retroviral reverse transcriptase. The cDNA is then amplified exponentially using PCR. This product can be quantified, cloned, used as a probe, or used as a template for in vitro transcription or sequencing.

Historically, steady state levels of individual RNAs have been measured by Northern blotting, nuclease protection assays, and in situ hybridization. These techniques, however, are limited by their sensitivity. Under optimal conditions, at least approximately 10,000 copies of an RNA transcript are required for detection by ribonuclease protection assay, the most sensitive non-PCR based mRNA detection and quantitation procedure (unpublished results and Lee et al., 1996). RT-PCR overcomes this limitation, and is capable of quantitating transcription down to single-transcript-per-cell sensitivity. In theory, a single copy of a cDNA can be detected by PCR, but approximately 100 copies is the practical lower limit of detection.

Relative RT-PCR is a popular method for the quantitative analysis of gene expression, despite an ongoing debate about its accuracy. The assumption behind relative RT-PCR is seductively simple: start with equal amounts of RNA from multiple samples, use identical RT-PCR conditions on each, and amplify the same target from each sample. When the products are analyzed, the amount of product from each reaction should be proportional to the abundance of the RNA transcript in the samples. The data obtained by relative RT-PCR is commonly expressed as "percent (or x-fold) difference" in the level of a specific RNA between otherwise equivalent RNA samples. In practice however, the reliability of results obtained by relative RT-PCR varies considerably, depending on the method and controls used. This kit was developed to provide reagents, controls and methodology to get reliable, relevant information about the relative abundance of different mRNA species in different sample RNAs.

B. Competimer™ Technology: Endogenous Standards for Relative RT-PCR

Sample to sample variability

Generally, there are two sources of sample to sample variability in relative RT-PCR experiments: differences caused by variations in the quantity or quality of the samples (e.g. partial degradation or the presence of contaminants), and random sample-to-sample variation (which includes user induced variation).

Unfortunately, random variability is a fact in PCR. The best way to minimize variability is to run duplicate samples and average the data. Random variability caused by operator error can be minimized by making cocktails of reagents.

Multiplex RT-PCR with an endogenous standard

To compensate for variations in RNA quality, initial quantitation errors, and random tube-to-tube variation in RT reactions and PCRs, "multiplex" RT-PCR can be performed. Multiplex PCR means using 2 (or more) primer sets in a single PCR - one set to amplify the cDNA of interest, and a second to amplify an invariant endogenous control. The level of product from the gene of interest is normalized against the product from the control.

ß-actin as an endogenous standard

ß-actin is a commonly used internal control; its expression level is often constant among samples taken from the same tissue. Although 18S ribosomal RNA is a more invariant control than ß-actin, it cannot be used for relative RT-PCR with poly(A) RNA. Researchers who have already done Northerns or other types of RNA analysis using ß-actin, may also choose ß-actin as a standard in RT-PCR studies for consistency.

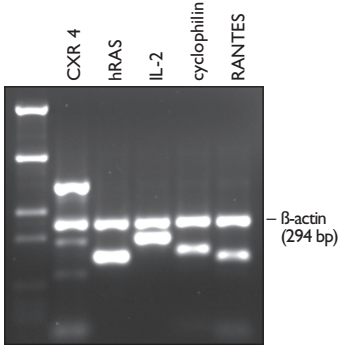

Figure 1. Relative RT-PCR using ß-actin internal standards

One µg of total mouse RNA was reverse transcribed using the Ambion RETROscript® kit. Aliquots of the cDNA were then subjected to relative RT-PCR using ß-actin internal standards with a ß-actin Primer:Competimer ratio of 4:6, and PCR primers for the indicated genes.

Using ß-actin as an internal standard for RT-PCR presents some specific challenges. First, the abundance of the actin transcript makes it difficult to multiplex with less abundant transcripts. This is complicated by the fact that every RNA under study has a unique expression level, making it impossible to predict the desired expression requirements for an internal standard. Using the ß-actin Competimers in this kit overcomes this problem (see the next section). A second limitation of using actin as an internal control is the presence of processed pseudogenes in the mammalian genome. A processed pseudogene is created when a cellular mRNA is reverse transcribed and integrated back into the genome. Because these integrations occur randomly, pseudogenes typically lack promoters and are not expressed. Since pseudogenes are created from mature RNA, they also lack introns. Actin pseudogenes will contribute to the PCR product in experiments using RNA that is contaminated with genomic DNA, regardless of whether the PCR primers are designed to flank introns (as are Ambion's). In practice this is not much of a problem; since there are many thousands of copies of actin message per cell, and less than 10 pseudogenes, their presence will not affect relative quantitation. If there is any residual DNA in the template RNA, however, the minus-RT control sample will make a product in PCR using the ß-actin PCR Primer Pair. If it is necessary, the RNA samples can be treated with DNase 1 to remove genomic DNA contamination.

The PCR primers and competimers in this kit were designed to have homology with human, mouse and rat ß-actin. They will probably also work with many other mammalian species. There are several actin mRNAs in mammalian cells that have 95–98% homology with ß-actin. Because the different actin messages are so similar, the ß-actin PCR Primer Pair and Competimers will function in PCR with several actin cDNA species in addition to ß-actin.

Competimer™ technology makes it feasible to use actin as a standard

Our Competimer™ technology can be used to modulate the amplification efficiency of a PCR template without affecting the performance of other targets in a multiplex PCR. Actin Primers and Competimers are supplied separately as 5 µM stocks of forward and reverse primers mixed in a 1:1 ratio. The Actin Competimers are modified at their 3' ends to block extension by DNA polymerase. By mixing Actin Primers with increasing amounts of Actin Competimers, the overall PCR amplification efficiency of actin cDNA can be reduced without the primers becoming limiting and without loss of relative quantitation.

C. Reagents Provided with the Kit and Storage Conditions

The kit should be stored in a non-frost-free freezer (supply is enough for 100 PCRs, 50 µL each).

Amount	Component	Storage
400 µL	Actin Primer Pair (5 µM)	–20°C
400 µL	Actin Competimers™ (5 µM) Modified oligonucleotides used in combination with Actin Primers. Allows actin RNA to be used as a versatile internal control.	–20°C
50 µL	Control Template RNA (0.5 µg/µL, total RNA from mouse liver)	below –70°C
1.75 mL	Nuclease-free Water	any temp*

* Store Nuclease-free Water at –20°C, 4°C, or room temp.

The Actin Primers and Competimers™ supplied with the kit are calibrated *as a pair* to generate a consistent RT-PCR signal. Pairs of Actin Primers and Competimers will have the same lot number. It is not advisable to mix Actin Competimers with Actin Primers from a different pair.

D. Required Materials not Included in the Kit

RT-PCR reagents:

- Thermostable DNA polymerase (recommended: Ambion SuperTaq™*, recombinant thermostable DNA polymerase, P/N AM2050, AM2052)
- Reagents for first strand cDNA synthesis (recommended: Ambion RETROScript® Kit P/N AM1710, or Cells-to-cDNA™ II P/N AM1722).
  Reagents needed include random 6- to 10-mers or oligo $dT_{(12-18)}$, reverse transcriptase, and reaction buffer.
- Thermal cycler

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General reagents:

- Disposable, RNase-free, polypropylene thin wall microfuge tubes and pipette tips
- Materials and equipment for analyzing products on native agarose gels
- Materials and equipment for analyzing products on polyacrylamide gels

E. Related Products Available from Applied Biosystems

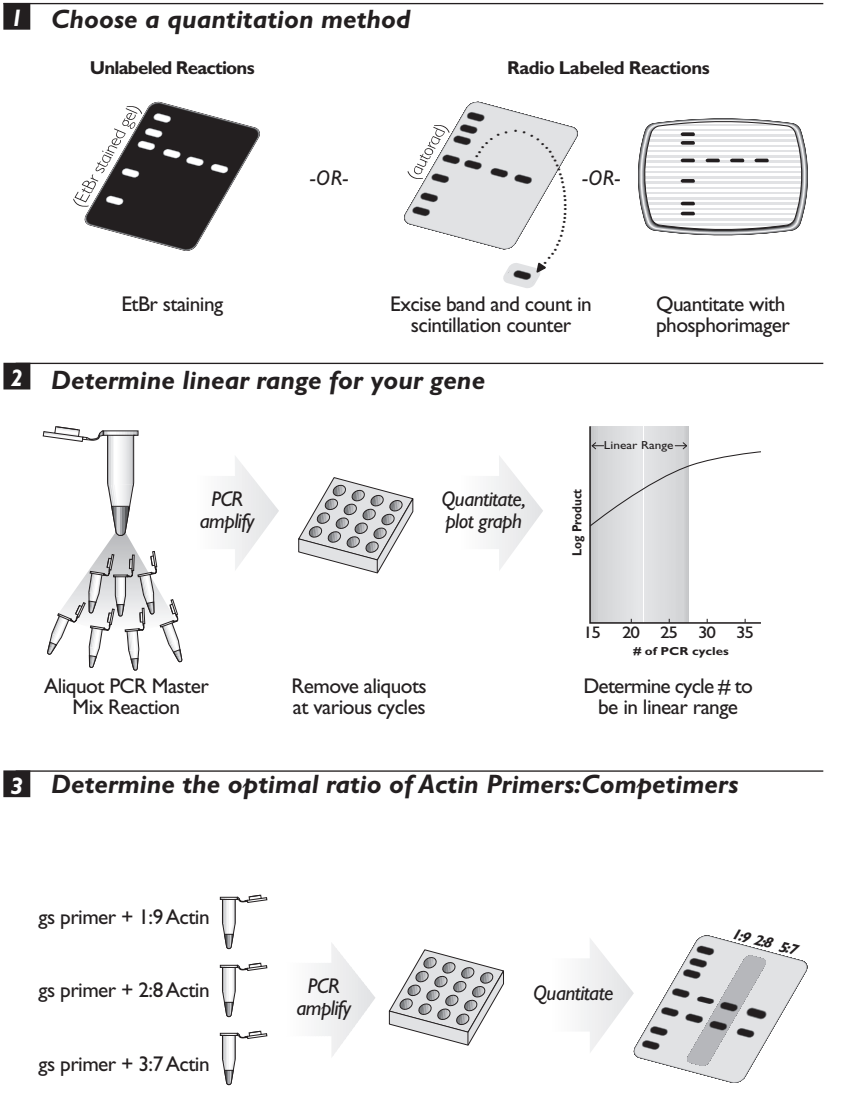
SuperTaq™ P/N AM2050, AM2052	Thermostable DNA Polymerase (includes 10X buffers and dNTPs)
RETROscript® Kit P/N AM1710	First strand cDNA synthesis kit for RT-PCR. When purchased with SuperTaq™, this kit provides reagents, controls and protocols for reverse transcription and PCR. Both oligo(dT) and random primers for cDNA priming are included, as is RNase inhibitor.
Cells-to-cDNA™ II Kit P/N AM1722, AM1723	The Cells-to-cDNA II Kit (patent pending) is designed for reverse transcription directly from mammalian cell lysate, without RNA isolation. It is ideal for synthesizing cDNA from small numbers of cells, numerous cell samples, or for labs that are not equipped for RNA isolation.
DNA-*free*™ Reagents P/N AM1906	DNase treatment and removal reagents. This product contains Ambion's ultra-high quality RNase-free DNase I and reaction buffer for degrading DNA. It is ideal for removing contaminating DNA from RNA preparations. A novel reagent for removing the DNase without the hassles or hazards of phenol extraction or alcohol precipitation is also included.
RNase-free Tubes & Tips see our web or print catalog	Ambion RNase-free tubes and tips are available in most commonly used sizes and styles. They are guaranteed RNase- and DNase-free. See our latest catalog or our website (www.ambion.com/prod/tubes) for specific information.
Electrophoresis Reagents see our web or print catalog	Ambion offers gel loading solutions, agaroses, acrylamide solutions, powdered gel buffer mixes, nuclease-free water, and RNA and DNA molecular weight markers for electrophoresis. Please see our catalog or our website (www.ambion.com) for a complete listing as this product line is always growing.

II. Experimental Design

A. General Guidelines for RT-PCR

This section describes, in detail, how to choose the best experimental conditions for relative RT-PCR experiments; this process is shown schematically in Figure 2.

Figure 2. Experimental Design for Relative Quantitative RT-PCR



1. Total RNA or poly(A) RNA

For RT-PCR applications, it is usually not necessary to isolate the polyadenylated fraction to use as template in the reverse transcription step, even for rare messages. This is because PCR can efficiently amplify targets even when they comprise only a very small proportion of the total cDNA produced in the RT.

2. RT primers

It makes little or no difference whether random hexamers or decamers, or oligo dT is used as primer in the reverse transcription reaction. When ordering primers for reverse transcription, Ambion recommends that a trityl-on, reverse phase cartridge purification be performed.

3. PCR primer design

For the most efficient design of PCR primers for RT-PCR applications, the use of primer design software is highly recommended. These programs are useful for designing primers free of features that contribute to the production of unwanted side products. They also provide useful information about optimal primer annealing temperature and other important parameters to consider in optimizing the thermocycle profile. If possible, PCR primers should be designed to avoid regions of high secondary structure, as these may inhibit the read-through activity of reverse transcriptase (Pallansch et al., 1990).

It is also advantageous to choose PCR primers that span at least one intron-exon boundary in the target mRNA, to prevent amplification of the target region from any contaminating DNA that may be present in the template RNA, or to at least yield a product that would be distinguishable (by its larger size) from the intended RT-PCR product. Sequence information, including the positions of intron-exon boundaries, can be found for many genes in the Genbank database.

Primers should be tested in RT-PCR alone before attempting the multiplex reaction. The PCR should produce a single, discrete product that does not comigrate with the product from the Actin Primers. Ambion has done multiplex RT-PCR with over 100 primer pairs. We have found that primers which perform well alone at a certain annealing temperature, frequently require a higher annealing temperature to maintain specificity in multiplex reactions. The required increase may be as much as 5°C, and it must be determined empirically.

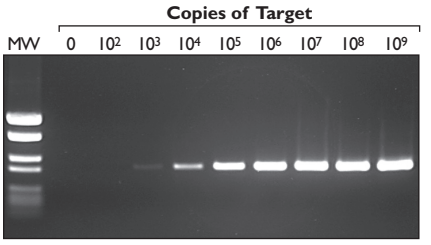
B. Quantifying PCR Products

A method to detect PCR products:

- should be sensitive
- should have a wide dynamic range
- must be quantifiable

Most researchers are familiar with evaluating PCR products on ethidium bromide (EtBr) stained agarose gels—some might even think their eyes are an analytical instrument sensitive enough to quantify EtBr

fluorescence. Unfortunately, EtBr staining is a poor method to quantify relative RT-PCR, primarily because the visualization of DNA on agarose gels with EtBr staining is relatively insensitive. The lower limit of detection of a band is about 5–10 ng. If 10 µL of a PCR is run in a gel, that means the minimum yield of the PCR necessary to visualize the product is 25–50 ng. If the upper limit of yield of the PCR at plateau is 500 ng, the dynamic range of the system (the range between the lower limit of detection and the top of the linear range) would be 1 log (see figure 3 on page 8). This means that if the level of expression of a target varies by more than one log, large errors in quantitation will occur. Switching from EtBr to alternate staining agents such as Syber Green (Molecular Probes) or silver staining would greatly increase the sensitivity of detection and would increase the dynamic range of the system.



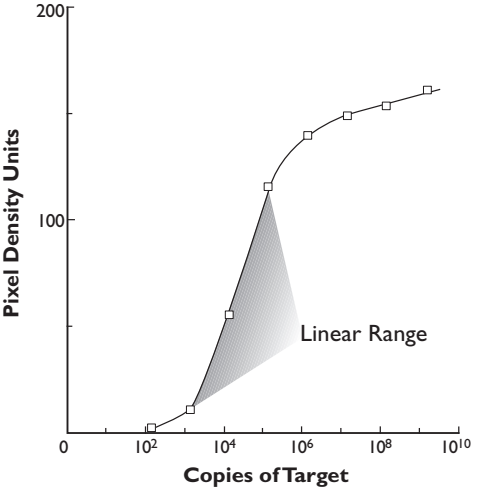


Figure 3. Limited Dynamic Range of Relative RT-PCR analysis by EtBr

Log dilutions of a synthetic IL-5 RNA was spiked into a constant amount of yeast RNA and amplified by RT-PCR for 30 cycles. Equal volumes of each sample were run on a 2% agarose EtBr gel. The image was captured electronically, and the bands were quantified using image analysis software. The limit of detection on this gel is 10^3 copies per sample, the reaction is out of linear range by 10^5 copies. Note that although the reaction is not quantitative past 10^5 copies, the yield continues to increase up to the sample containing 10^9 copies.

1. Quantifying the signal on stained gels

If the dynamic range of EtBr (or other) fluorescence is acceptable for your application, you must have access to a gel documentation system and associated software for quantitation. Typically the digital cameras and software applications (e.g. NIH Image), are 8 bit. This means they have a maximum theoretical dynamic range of 256 'levels of signal'. This can be expanded by capturing data at several exposure times and factoring in a correction factor. For example, an image can be captured with a 0.2 second exposure and a 2 second exposure to keep both strong and weak bands within the linear range of the camera. A correction factor of 0.1X is then applied to the signals obtained using the longer exposure.

Because PCR produces both double- and single-stranded products, amplification products frequently appear as doublets on nondenaturing gels. These products, easily detected when the products are labeled, are not as obvious on EtBr stained gels since the single stranded material does not stain well. In any case, this is not a problem for quantitation as both bands can be quantified together. Alternatively, the products can be denatured and run as single-stranded DNA on a denaturing polyacrylamide gel.

2. Is my gel documentation system quantitative?

A good test of a gel documentation system's ability to quantify signal intensity is to run a molecular weight marker (made by restriction enzyme digestion) and quantify the bands. The intensity of a band should be directly proportional to its molecular weight. For example, if λ*Hin*d III marker is run and the bands are quantified, the 9.4 kb band should measure 1.42 times as intense as the 6.6 kb band. This simple experiment will reveal a great deal about both the system and its ability to provide reliable information. Alternatively, you can run and quantify a serial dilution of a PCR product.

3. Increasing the Dynamic Range: Trace Labeling with [^{32}P] dNTP

There are two ways to label a PCR with ^{32}P. The PCR primers can be end labeled with [γ-^{32}P] ATP using T4 polynucleotide kinase, or [α-^{32}P] dATP (or dCTP) can be added to the PCR. The latter technique is a bit less sensitive, but is technically much simpler. PCRs are simply spiked with a small amount (e.g. 1 µL, 10 µCi of 3000 Ci/mM) of [α-^{32}P] dATP (or dCTP) and the reactions are run as usual. For maximum resolution and ease of downstream handling, the products are resolved on a 6% polyacrylamide gel.

Quantification using a phosphorimager

The gel is dried down and the products are quantified using a phosphorimager. The advantage of quantifying radio-labeled products is an extraordinary increase in sensitivity and dynamic range (a phosphorimager has a dynamic range of 6.5 x 10^4).

Quantification using a scintillation counter

The low tech approach to quantifying PCR products with radioactivity is to run the products in an acrylamide gel, transfer the gel to blotting paper, wrap in plastic wrap, and image the wet gel by autoradiography. Next, the gel is lined up with the autorad, the bands are excised with a scalpel, and counted in a scintillation counter. Although tedious, this method yields very accurate results.

4. Trace labeling with nonisotopically modified nucleotides

An alternative approach to isotopic analysis is to incorporate modified deoxyribonucleotides during the PCR. Well established systems exist for the detection and quantitation of biotin, digoxygenin, and fluorescein. Since the PCR can be inhibited by using too high a ratio of modified nucleotide, a reasonable approach is to use a 10:1 (or lower) ratio of standard dNTP:modified dNTP. The PCR products are run in a gel

and transferred to a nylon membrane (by capillary or electroblotting for agarose, or electroblotting for acrylamide). The membrane is then taken through a detection protocol that ends with the application of a chemiluminescent substrate. The products are quantified using a phosphorimager or similar device.

It is also possible to incorporate fluorescent-tagged dNTPs during the PCR. The products are then quantified on the appropriate apparatus.

C. Determining the Linear Range for your RQ RT-PCR

1. Amplification efficiency

The amplification efficiency of a specific PCR is dependent on both physical parameters that do not change between different reactions and on the quality of the reagents. For example, the cycling performance of the thermal cycler, the thermal conductivity of the heat block, and the fit of the reaction tubes in the thermal cycler do not vary between samples but these factors impact amplification efficiency. The quality of the thermostable polymerase (specific activity, half-life at 94°C, and intrinsic error rate) also contributes to overall amplification efficiency. The biggest variables affecting amplification efficiency, however, are sample and primer specific. These include primer performance, amplicon length, and template quality.

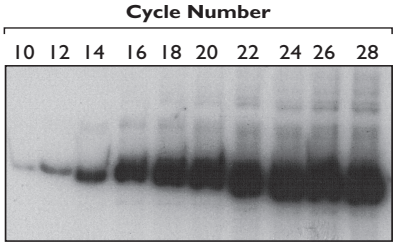
2. Relative RT-PCR must be quantified in the linear range of PCR

Reaction products accumulate during a PCR at a rate dependent on the amplification efficiency. The linear range of the reaction is defined as the period of the PCR in which the amplification efficiency is at its maximum and remains constant over a number of cycles. In other words, graphing "cycles vs. product" produces a straight line. At some point during the reaction, the amplification efficiency falls and the rate of product accumulation slows or "plateaus".

To obtain meaningful results, relative RT-PCRs must be terminated for product quantification when all the samples are in the linear range of amplification. When the target varies in abundance between samples, the point at which the reaction plateaus will be different for each sample. For example, a PCR in which the initial abundance of the target is 10 fold higher than another will plateau sooner.

3. Determining linear range

As described above, a PCR will remain in linear range for only a limited number of cycles. It is therefore necessary to determine this range of cycles for all PCR products being measured. An example of such an experiment is shown in Figure 4 on page 11, and the procedure for determining the linear range of your samples is in the following section. Briefly, this involves choosing the RNA sample in which the target is expected to be most abundant, or pooling several RNA samples, and assembling 10 identical PCRs. The PCR is then split into 10 aliquots, and the reaction is subjected to PCR. Aliquots are then removed every 2 cycles, and the results are quantified and plotted.



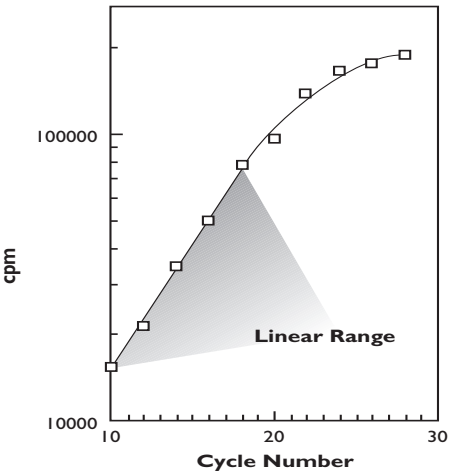


Figure 4. Determining Linear Range

A PCR master mix was prepared including 10 µL [α-^{32}P]dCTP in addition to the normal reaction components. The master mix was split into 10 aliquots which were then subjected to PCR. Aliquots were removed from the thermocycler at the indicated cycle numbers and resolved by electrophoresis on a 5% polyacrylamide/urea gel. The products were quantitated with a Bio-Rad Molecular Imager. Cycle number is plotted against the log of the signal and a straight line is obtained for samples in linear range of amplification.

4. Experiment setup

Conditions for every primer pair/target must be empirically determined. This is dependent on both amplification efficiency and target abundance. Two questions will be answered by this experiment:

- How sensitive is the detection system? In other words, how few PCR cycles produce a reliably detectable product.
- How many PCR cycles are required to reach plateau.

The larger the difference in cycle number between these two values, the wider the dynamic range of the detection system. Once the linear range is established, pick a cycle number in the middle of the linear range to use in future experiments to give a maximum range on both sides of a 'typical sample'. The only requirement for doing this first experiment is to choose an appropriate amount of RNA to use in the RT reaction.

a. Assemble RT reaction on ice in a thin wall PCR tube:

Amount	Component
up to 2.5 µg*	total RNA
4 µL	dNTP mix: 2.5 mM each
2 µL	first strand primers†(50 µM)
to 16 µL	Nuclease-free Water

* much less RNA can be used if the target is abundant or if RNA is limiting.

† 0.2–5 µM or 20–300 ng random primers (6–10mers) or oligo $dT_{(12\text{-}18)}$ per reaction. Concentrations of first-strand primers within this range typically work equally well in reverse transcription.

IMPORTANT

If the RNA has any Mg^{++} in the buffer (for example, from DNase reaction buffer), omit the heating step or the RNA will be degraded.

b. Denature RNA secondary structure.
Mix, spin briefly in microfuge, heat 3 min. at ~70–85°C.
Remove tube(s) to ice; spin briefly, place on ice.

c. Add the remaining RT components:
Mix gently, spin briefly.

Amount	Component
2 µL	10X RT-PCR Buffer*
1 µL	(optional) RNase inhibitor
100 units	M-MuLV RT

* 100 mM Tris-Cl pH 8.3, 500 mM KCL, 15 mM $MgCl_2$

d. Incubate at 42°C for 1 hour.

e. Store reaction at –20°C or proceed to amplification step.

f. Set up the PCRs on ice.
On ice, make a PCR cocktail for 10 samples (with 10% overage)

Amount	Component
11 µL	RT reaction (from c above)
55 µL	10X PCR Buffer
44 µL	2.5 mM dNTPS
44 µL	Gene specific primer pair (5 µM each) -final concentration of 0.4 µM each
2.75 µL	thermostable DNA polymerase (4–5 U/µL)
to 545 µL	Nuclease-free Water
5.5 µL	[α-^{32}P] dCTP (10 µCi/µL)

Split the cocktail into 10 identical PCR tubes (50 µL each).

IMPORTANT
Ambion recommends a hot start for PCRs. At a minimum, assemble reactions on ice, preheat thermocycler to 94°C, and then place the tubes in the thermocycler.

g. Do the PCR
Program the PCR machine for 35 cycles of the following profile:

94°C	30 sec
annealing temp.*	30 sec
72°C	30 sec

* Use the annealing temperature that works well for PCR of your gene of interest, the Actin Primers work best with annealing temperatures from 54-62°C, but they work almost as well using annealing temperatures from 63-68°C.


NOTE

Thermal cyclers with very short ramp times, may require slightly longer incubation times, whereas machines with virtually no ramp time, such as Stratagene's RoboCycler®, will probably require 1 minute at each temperature in the cycle for good results.

h. Remove samples at different cycle numbers.
Remove samples and place on ice after each odd numbered cycle starting with cycle 15, ending with cycle 33.

i. Analyze results by 6% PAGE.
Native or denaturing gels can be used, recipes are found in section V.A on page 22. If nondenaturing gels are used, the bands may appear as doublets because PCRs often contain both double- and single-stranded products.

Add loading dye to 10 µL of each reaction, (denature samples at 95°C, 3 min if using denaturing gels), and run samples on a 6% acrylamide gel.

j. Quantify results.
Transfer gel to blotting paper (e.g. Whatman 3mm), dry the gel, and quantify product using a phosporimager, or x-ray film and a densitometer. Alternatively, dry the gel, expose to x-ray film, localize bands, excise them from the gel, and count in a scintillation counter.

k. Graph the results, and select the best cycle number.
Plot the log of the signal on the Y-axis vs. the cycle number on the X-axis. The ideal cycle number for subsequent experiments will be one that is squarely in the middle of the detectable linear range. It is possible that 15 cycles is well above the threshold of detection. If so, you can choose a cycle number closer to 15 cycles.

D. Determine the Optimal Ratio of Actin Primers to Competimers

Since they compete for available resources, it is critical that the control target (actin) be amplified from the RT-PCR at a level roughly similar to the amplicon under study. If one target is present at a significantly higher concentration than the other and both are amplified at a similar efficiency, competition for reagents in the PCR will result in a loss of exponential amplification for the template of lower abundance. If two targets are present at similar levels, but one is amplified at a higher efficiency, a similar result will occur. (Because of competitive interference, the ability to obtain quantitative information will be lost.) The following experiment will determine what ratio of primers to Competimers are needed to have both the target-of-interest, and the actin control target amplify to give similar yields of product.

A 2:8 ratio of Actin Primers to Competimers is appropriate for most genes if the experiment will have a relatively large dynamic range. A 1:9 or lower ratio may be needed for extremely rare messages, and a 3:7 ratio may work best for relatively abundant messages. If less than a 1:9 ratio is needed, Ambion recommends doubling the amount of Actin Primers and Competimers in the reaction (i.e. use 8 µL of the Primer:Competimer mixture in step *II.D.2. Set up the PCR cocktail* on

page 14 instead of 4 µL). This is necessary to maintain a sufficient concentration of Actin Primers in the reaction, but the kit reagents will not be sufficient for 100 PCRs. To prevent primers from becoming limiting in the PCR, use the smallest number of PCR cycles that produce detectable and quantifiable products.

NOTE

If it is necessary to do nested PCR for a very rare target, omit the Actin Primers from the first PCR and add them to only the second reaction.

If the experiment will be quantified using EtBr, or if using a 2:8 ratio yields only actin product, the following experiment is suggested to pinpoint the ideal Actin Primer to Competimer ratio. Repeat this pilot experiment for every new RT-PCR primer pair, since the starting abundance of other cDNA targets is initially unknown. Once the proper mix of primers:Competimers is established, the actin product can be used to correct for sample-to-sample variation in the amount of total RNA used as template and for tube-to-tube variations in the PCR step.

IMPORTANT

The Actin Primers and Competimers™ supplied with this kit are calibrated as a pair to generate a consistent RT-PCR signal. Pairs of Actin Primers and Competimers will have the same lot number. It is not advisable to mix Actin Competimers with Actin Primers from a different pair, because if you do, you may need to repeat the experiment that determines which ratio of Actin Primer:Competimer is best in your system.

1. Prepare Actin Primer: Competimer™ mixtures

Generally, messages with the following abundance levels require the Actin Primer:Competimer ratios shown:

Transcript Abundance	Actin Primer: Competimer Ratio
Abundant transcripts	3:7
Moderately expressed transcripts	2:8
Rare transcripts	1:9
Extremely rare transcripts	<1:9

Prepare the Primer:Competimer mixtures as follows:

Actin Primer:Competimer ratio			
1:9	2:8	3:7	
9 µL	8 µL	7 µL	Actin Competimers
1 µL	2 µL	3 µL	Actin Primer Pair

2. Set up the PCR cocktail

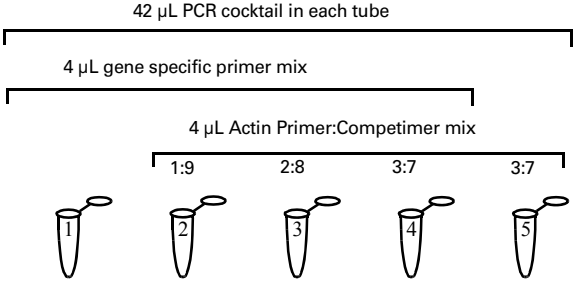
On ice: using an aliquot of an RT reaction performed as shown in steps C.4.a–d (page 11), make a PCR cocktail for 5 samples (with 10% overage to allow for pipetting error).

NOTE

A hot start for multiplex RT-PCRs is recommended. At a minimum, assemble reactions on ice, preheat thermocycler to 94°C, and then place the tubes in the thermocycler.

Amount	Component
5 µL	template (RT reaction)
27.5 µL	10X PCR Buffer
22 µL	2.5 mM dNTPs
1.375 µL	thermostable DNA polymerase
175 µL	Nuclease-free Water
2.5 µL	[α–^{32}P] dCTP

Aliquot 42 µL of the PCR cocktail to 5 tubes labeled 1–5, and add gene specific primers and Actin Primer and Competimer mixtures to the tubes as shown below:



3. Perform the PCR

Use the temperatures and the number of cycles determined in the linear range determination experiment (section II.C), and assess the results by gel electrophoresis.

4. Choose the best ratio

The lane in which the level of actin product is most similar to the level of the gene specific product contains the correct ratio of Actin Primer to Competimer for use with the gene specific primers tested. It is possible that more than one (or all three) Actin Primer:Competimer mix will meet these criteria. The smaller the dynamic range of your detection method, the more critical this experiment will be. Figure 5 shows a quantitative RT-PCR experiment where different ratios of 18S primers and competimers were compared, the results using ß-actin primers and competimers should be similar.

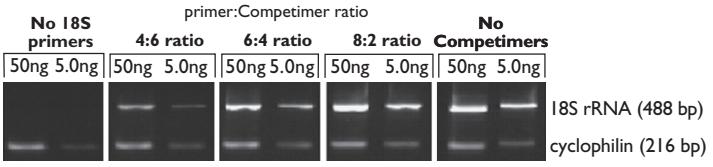


Figure 5. Example of different primer:Competimer ratios in relative quantitative RT-PCR for cyclophilin

In this example, the 4:6 primer:Competimer ratio is the best because the staining of the 18S rRNA target is equivalent to that of the cyclophilin target.

III. Relative Quantitative RT-PCR

A. Do Your Experiment

At this point, cycling parameters, linear range, and the optimal Actin Primer:Competimer ratio have all been established. Use this information to do relative, quantitative RT-PCR experiments using the reaction conditions identified in the preliminary work.

Keep the following recommendations in mind:

- Where possible, set up an n + 10% (overage to allow for pipetting error) cocktail for shared components. This will help reduce sample to sample variation.
- Always set up PCRs and cocktails on ice and preheat the thermal cycler before placing your sample tubes in the machine.

1. Assemble RT reaction on ice in a thin wall PCR tube:

Amount	Component
up to 2.5 µg*	total RNA
4 µL	dNTP mix: 2.5 mM each
2 µL	first strand primers†(50 µM)
to 16 µL	Nuclease-free Water

* much less RNA can be used if the target is abundant or if RNA is limiting.

† 0.2–5 µM or 20–300 ng random primers (6–10mers) or oligo $dT_{(12\text{-}18)}$ per reaction. Concentrations of first-strand primers within this range typically work equally well in reverse transcription.

IMPORTANT

If the RNA has any Mg^{++} in the buffer (for example, from DNase reaction buffer), omit the heating step or the RNA could be degraded.

2. Denature RNA secondary structure

Mix, spin briefly in microfuge, heat 3 min. at ~70–85°C.

Remove tube(s) to ice; spin briefly, place on ice.

3. Add the remaining RT components and mix:

Amount	Component
2 µL	10X RT-PCR Buffer*
1 µL	(optional) RNase inhibitor
100 units	M-MuLV RT

* 100 mM Tris-Cl pH 8.3, 500 mM KCL, 15 mM $MgCl_2$

4. Incubate at 42°C for 1 hour

After the 1 hr incubation at 42°C, store the RT products at –20°C or proceed to amplification step

5. Prepare the PCR cocktail on ice

The following example shows the set up for a PCR cocktail for 10 samples (with 10% overage). Assemble the following on ice:

Amount	Component
55 µL	10X PCR Buffer
44 µL	2.5 mM dNTPS
44 µL	Actin Primer:Competimer mixture (at the optimal ratio determined in section D.4 on page 15)
44 µL	Gene specific primer pair (5 µM each) -final concentration of 0.4 µM each
2.75 µL	thermostable DNA polymerase (4–5 U/µL)
to 534 µL	Nuclease-free Water
5.5 µL	[α-^{32}P] dCTP (10 µCi/µL)

6. Aliquot the PCR cocktail and add 1 µL cDNA to each tube

a. Aliquot the PCR cocktail into 10 PCR tubes on ice: 49 µL per tube.

b. Add 1 µL of the RT reaction (from step 4 above) to each tube

7. Do the PCR

IMPORTANT

Ambion recommends a hot start for PCRs. At a minimum, assemble reactions on ice, preheat thermocycler to 94°C, and then place the tubes in the thermocycler.

Using the cycle number determined in the pilot experiment, program the thermocycler as indicated below:

94°C	30 sec
annealing temp.*	30 sec
72°C	30 sec

* Use the annealing temperature that works well for PCR of your gene of interest, the Actin Primers work best with annealing temperatures from 54–62°C, but they work almost as well using annealing temperatures from 63–68°C.

B. Processing the Data

When you are quantifying PCR products, for each sample divide the signal obtained for the gene specific amplicon by the signal obtained for the actin amplicon. This will yield a corrected relative value for the gene specific product in each sample. These values may be compared between samples for an estimate of the relative expression of target RNA in the samples.

Keep in mind that relative RT-PCR only provides an estimate of the relative changes in gene expression between samples. If an exact value is needed, use competitive quantitative RT-PCR.

IV. Troubleshooting RT-PCR

A. Low Yield of RT-PCR Product

Low yield of RT-PCR products can be due to many factors. Degraded or impure RNA can limit the efficiency of the RT reaction and reduce yield. Poor primer design or non-optimized cycling parameters or reaction conditions can limit the yield in a PCR. Problems in the PCR step can also result in co-amplification of non-specific products and poor reproducibility of results. If the desired PCR products are not produced in adequate quantity and/or purity, the following suggestions may improve the reaction.

1. Do the positive control

Verify that the RT and PCR reagents and equipment are OK

The QuantumRNA ß-actin Internal Standards come with an aliquot of Control Template RNA from mouse liver. You can use this RNA as a control in an RT-PCR to verify that your reverse transcription and PCR reagents, as well as your thermal cycler are performing properly.

Do the reverse transcription as shown in section II.C.4.a–e (starting on page 11) using 2 µL of the Control Template RNA. Make three 10-fold dilutions of the RT reaction and do four 30 cycle PCRs; one on 1 µL of the undiluted RT reaction and one each on 1 µL of each dilution. The PCR master mix is shown below:

IMPORTANT

Ambion recommends a hot start for PCRs. At a minimum, assemble reactions on ice, preheat thermocycler to 94°C, and then place the tubes in the thermocycler.

Amount	Component
22 µL	10X PCR buffer
17.6 µL	Actin Primer Pair
4.4 U	thermostable DNA polymerase
to 216 µL	Nuclease-free Water

Program the PCR machine for 30 cycles of the following profile:

94°C - 30 sec
57°C - 30 sec
72°C - 30 sec

NOTE

Thermal cyclers with very short ramp times, may require slightly longer incubation times, whereas machines with virtually no ramp time, such as Stratagene's RoboCycler®, will probably require 1 minute at each temperature in the cycle for good results.

Run 10 µL of the PCR on a 2% agarose gel stained with ethidium bromide. In our experience, the actin PCR amplification (without Competimer) is very robust. The 294 bp actin product should be seen in all four samples.

Verify that your RNA is OK

The Actin Primers can also be used with your RNA to verify that it is competent in RT-PCR. To set up this control, do a reverse transcription reaction using 1 µg of your RNA; follow the instructions in sections II.C.4.a–e (starting on page 11). Use 1 µL of the RT in PCR:

IMPORTANT

Ambion recommends a hot start for PCRs. At a minimum, assemble reactions on ice, preheat thermocycler to 94°C, and then place the tubes in the thermocycler.

Amount	Component
1 µL	RT reaction
5 µL	10X PCR buffer
4 µL	2.5 mM dNTPs
4 µL	Actin Primer Pair
1 U	thermostable DNA polymerase
to 50 µL	Nuclease-free Water

Program the thermal cycler for 30 cycles of the following profile:

94°C - 30 sec
57°C - 30 sec
72°C - 30 sec

Thermal cyclers with very short ramp times, may require slightly longer incubation times, whereas machines with virtually no ramp time, such as Stratagene's RoboCycler®, will probably require 1 minute at each temperature in the cycle for good results.

Run 10 µL of the PCR on a 2% agarose gel stained with ethidium bromide. If the 294 bp actin product is seen, it indicates that the experimental RNA is amplifiable by RT-PCR, if no product is seen, try cleaning it up as discussed in the next section.

2. Inhibitors of reverse transcription and/or PCR in the RNA

Possible inhibitors of reverse transcriptase include RNases and contaminants such as guanidinium, proteinase K, or alcohol carried over from the RNA isolation procedure. Contaminants can usually be removed by phenol extraction and re-precipitation of the RNA, followed by washing the pellet with 70% ethanol. To remove all traces of alcohol after precipitation, re-spin the tube for a few seconds and remove the residual supernatant with a finely drawn-out Pasteur pipette or a fine-bore needle. Allow the pellet to dry thoroughly before resuspending it.

B. Spurious RT-PCR Products

Non-specific priming

Sometimes unexpected products in an RT-PCR come from non-specific priming of unrelated cDNA sequences during the amplification. Raising the stringency of the PCR by increasing the annealing temperature can often improve results.

Unexpected products

Some other causes of unexpected bands are alternatively spliced transcripts, amplification from contaminating genomic DNA, or priming off a transcript from a related gene. If primers are designed to flank an intron, PCR products made from contaminating genomic DNA will typically be a different size than products from cDNA. As discussed in section *I.B. ß-actin as an endogenous standard* on page 2, a processed (introns spliced out) pseudogene will yield the correct size fragment from genomic DNA using the Actin Primer Pair for PCR.

Identifying PCR products amplified from contaminating DNA

The surest way to know whether RT-PCR products are amplified from genomic DNA or not is to run a "minus-RT" control. That is, omit the reverse transcriptase from the RT reaction, but otherwise do the RT-PCR as usual. Any product from a reaction without reverse transcriptase must be amplified from a DNA template. The recommended course of action is to treat the RNA preparation with DNase (Ambion DNA-*free*™ P/N AM1906), and then to inactivate the enzyme.

If the problem bands are RT specific, they may represent alternatively spliced forms of your transcript or amplification of a fragment from a related transcript. Cloning and sequencing of the fragments would resolve this question. Alternatively, primers can be designed to a unique region of the transcript (e.g. the 3ə untranslated region).

Unexpected primer interactions

In multiplex PCR there is always the possibility of unpredicted interaction between Actin and gene specific primers. This may be manifested as unexpected bands, specific to PCRs in which both primer pairs are present. Ambion has tested the Actin Primers in multiplex RT-PCR with many gene specific primer pairs. We sometimes observe that primers which function perfectly at one annealing temperature require a higher temperature to function when used in multiplex PCR with actin. We suggest increasing the annealing temperature by two degree intervals and assessing the products after 30–35 cycles on an EtBr stained agarose gel.

C. RT-PCR False Positives and Contamination

That the sensitivity of PCR is a double-edged sword is best illustrated by the following example:

> A PCR with a yield of 2 µg of a 250 bp product, if spilled into a 60,000 liter swimming pool, would result in a contamination of 121 copies per µL of pool water. In more practical terms, in each picoliter of the above PCR there are 3.65×10^5 copies of the product.

Careful laboratory practices are necessary to avoid contaminating reactions with PCR products. A separate area should be used as a pre-PCR workbench. No concentrated DNA solutions (PCR products, plasmid prep, etc.) should be allowed in the area. A set of pipetmen should be reserved for setting up reactions and barrier tips should be used to avoid contaminating primers, enzymes or nucleotide solutions. Completed PCRs should not be stored in the same location as PCR reagents. If negative controls start routinely turning up positive, more stringent steps may be taken to control contamination (see Yap et al., 1994).

V. Appendix

A. Recipes

1. 6% denaturing acrylamide gel mix

6% acrylamide /8M urea gel

15 mL is enough gel solution for one 13 x 15 cm x 0.75 mm gel

for 15ml	Component
7.2 g	urea (high quality)
1.5 mL	10X TBE
2.25 mL	40% acrylamide (acryl: bis-acryl = 19:1)
to 15 mL	dH_2O

Stir at room temp. until urea is completely dissolved, then add:

120 µL	10% ammonium persulfate in dH_2O
16 µL	TEMED

Mix briefly after adding the last 2 ingredients, which will catalyze polymerization, then pour gel immediately.

Gel set up

- Follow the manufacturers instructions for the details of attaching gels to the running apparatus.
- Use 1X TBE as the gel running buffer.
- It is very important to rinse the wells of urea-containing gels immediately before loading the samples.

Electrophoresis conditions

Gels should be run at about 20 V/cm gel length; for 13 cm long gel this will be about 250 V. Alternatively, denaturing acrylamide gels of this size can be run at ~25 mAmp, constant current.

2. 1–2X denaturing gel loading buffer

This gel loading buffer is also available from Ambion; it is called NorthernMax® Gel Loading Buffer II, P/N AM8546G, AM8547

Concentration	Component
95 %	formamide
0.025 %	xylene cyanol
0.025 %	bromophenol blue
18 mM	EDTA
0.025 %	SDS

3. 6% non-denaturing acrylamide gel mix

15 mL is enough gel solution for one 13 x 15 cm x 0.75 mm gel

for 15ml	Component
1.5 mL	10X TBE
2.25 mL	40% acrylamide (acryl:bis-acryl = 19:1)
to 15 mL	dH_2O

Stir at room temp, then add:

120 µL	10% ammonium persulfate in dH_2O
16 µL	TEMED

Mix briefly after adding the last two ingredients, which will catalyze polymerization, then pour gel immediately.

Gel set up

- Follow the manufacturers instructions for the details of attaching gels to the running apparatus.
- Use 1X TBE as the gel running buffer.

IMPORTANT

Do not heat the samples before loading.

Electrophoresis conditions

Non-denaturing gels must be run slowly to avoid heat denaturation of the samples. The voltage should be set to ~8.3 V/cm; a 13 x 15 cm x 0.75 mm gel should be run at about 100 V for ~2–3 hours.

4. 6X non-denaturing gel loading buffer

Concentration	Component	for 10 mL
37%	glycerol (100%)	3.7 mL
0.025%	bromophenol blue	2.5 mg
0.025%	xylene cyanol	2.5 mg
20 mM	1 M Tris-HCl, pH 8	200 µL
5 mM	500 mM EDTA	100 µL
	nuclease-free water	to 10 mL

Alternatively, Ambion offers an all-purpose Gel Loading Solution for native gels, P/N AM8556; this 10X solution is rigorously tested for nuclease contamination and functionality.

B. References

Lee P, Washer L, Law D, Boland CR, Horon I, and Feinberg A (1996) Limited up-regulation of DNA methyltransferase in human colon cancer reflecting increased cell proliferation. Proc. Natl. Acad. Sci. USA **93**: 10366–10370.

Pallansch L, Beswick H, Talian J, and Zelenka P (1990) Use of an RNA folding algorithm to choose regions for amplification by the polymerase chain reaction. Analyt. Biochem. **185**: 57–62.

Yap EPH, Lo Y-MD, Fleming KA, and McGee JO'D (1994) in PCR Technology, Griffen HG and Griffen, AM (eds.), 249–258

C. Quality Control

Nuclease testing

Relevant kit components are tested in the following nuclease assays:

RNase activity
Meets or exceeds specification when a sample is incubated with labeled RNA and analyzed by PAGE.

Nonspecific endonuclease activity
Meets or exceeds specification when a sample is incubated with supercoiled plasmid DNA and analyzed by agarose gel electrophoresis.

Exonuclease activity
Meets or exceeds specification when a sample is incubated with labeled double-stranded DNA, followed by PAGE analysis.

D. Safety Information

Chemical safety guidelines

To minimize the hazards of chemicals:

- Read and understand the Material Safety Data Sheets (MSDS) provided by the chemical manufacturer before you store, handle, or work with any chemicals or hazardous materials.
- Minimize contact with chemicals. Wear appropriate personal protective equipment when handling chemicals (for example, safety glasses, gloves, or protective clothing). For additional safety guidelines, consult the MSDS.
- Minimize the inhalation of chemicals. Do not leave chemical containers open. Use only with adequate ventilation (for example, fume hood). For additional safety guidelines, consult the MSDS.
- Check regularly for chemical leaks or spills. If a leak or spill occurs, follow the manufacturer's cleanup procedures as recommended on the MSDS.
- Comply with all local, state/provincial, or national laws and regulations related to chemical storage, handling, and disposal.

About MSDSs

Chemical manufacturers supply current Material Safety Data Sheets (MSDSs) with shipments of hazardous chemicals to new customers. They also provide MSDSs with the first shipment of a hazardous chemical to a customer after an MSDS has been updated. MSDSs provide the safety information you need to store, handle, transport, and dispose of the chemicals safely.

Each time you receive a new MSDS packaged with a hazardous chemical, be sure to replace the appropriate MSDS in your files.

Obtaining the MSDS

To obtain Material Safety Data Sheets (MSDSs) for any chemical product supplied by Applied Biosystems or Ambion:

- At **www.appliedbiosystems.com**, select **Support**, then **MSDS**. Search by chemical name, product name, product part number, or MSDS part number. Right-click to print or download the MSDS of interest.
- At **www.ambion.com**, go to the web catalog page for the product of interest. Click **MSDS**, then right-click to print or download.
- E-mail (MSDS_Inquiry_CCRM@appliedbiosystems.com) or telephone (650-554-2756; USA) your request, specifying the catalog or part number(s) and the name of the product(s). We will e-mail the associated MSDSs unless you request fax or postal delivery. Requests for postal delivery require 1–2 weeks for processing.

For the MSDSs of chemicals not distributed by Applied Biosystems or Ambion, contact the chemical manufacturer.