

USER GUIDE

**ambion**<sup>®</sup>  
by *life* technologies<sup>™</sup>

# QuantumRNA<sup>™</sup> 18S Internal Standards

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# About This Guide

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 **WARNING! ABBREVIATED SAFETY ALERTS.** Hazard symbols and hazard types specified in procedures may be abbreviated in this document. For the complete safety information, see the “Safety” appendix in this document.

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**IMPORTANT!** Before using this product, read and understand the information the “Safety” appendix in this document.

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## Purpose

The *QuantumRNA™ 18S Internal Standards User Guide* provides detailed procedures, reference information, and troubleshooting for the kit.

## User attention words

Five user attention words may appear in this document. Each word implies a particular level of observation or action as described below:

**Note:** Provides information that may be of interest or help but is not critical to the use of the product.

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**IMPORTANT!** Provides information that is necessary for proper instrument operation or accurate chemistry kit use.

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 **CAUTION!** Indicates a potentially hazardous situation that, if not avoided, may result in minor or moderate injury. It may also be used to alert against unsafe practices.

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 **WARNING!** Indicates a potentially hazardous situation that, if not avoided, could result in death or serious injury.

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 **DANGER!** Indicates an imminently hazardous situation that, if not avoided, will result in death or serious injury.

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Except for IMPORTANTs, the safety alert words in user documentation appear with an open triangle figure that contains a hazard symbol. These hazard symbols are identical to the hazard symbols that are affixed to the instrument. See the “Safety” appendix for descriptions of the symbols.



# QuantumRNA™ 18S Internal Standards

## Introduction

### 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.

### 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.

## Ribosomal RNA as an endogenous standard

Theoretically, an ideal endogenous standard would be one whose expression does not vary during the cell cycle, between cell types, or in response to the experimental treatment that one wishes to examine. At Life Technologies, we have found that even the commonly used “invariant” standard controls, such as  $\beta$ -actin, vary significantly from tissue to tissue as analyzed by Northern analysis (see figure 1 on page 8). Additionally, the mRNA component of total RNA can vary from 2% to 5%, depending on the tissue (unpublished observations). Therefore, even if a transcript were truly invariant (as expressed as a percentage of mRNA), it would still vary when considered as a percent of total RNA when one analyzes RNA samples from different tissue sources.

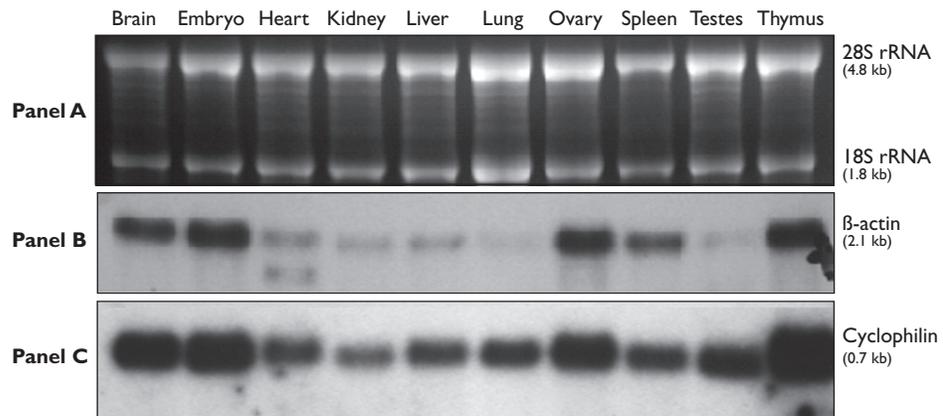
In RT-PCRs, input RNA is usually quantified by spectrophotometer. Since the majority of the RNA is rRNA, the level of rRNA remains essentially constant from sample to sample. Thus, in principle, rRNA should be the best internal control for RT-PCR. However, the abundance of rRNA is a major limitation to its utility as a control, since an endogenous control must be in the same linear range as the RNA under study when amplified under the same conditions. This is complicated by the fact that every RNA under study has a unique expression level. Thus, it is impossible to predict the desired expression requirements for an internal standard.

**Figure 1 Variation of  $\beta$ -actin and Cyclophilin expression in various mouse tissues.**

Panel A - Denaturing agarose gel of 3  $\mu$ g each of mouse tissue total RNA; stained with ethidium bromide.

Panel B - Northern blot hybridized with a  $\beta$ -Actin antisense RNA probe using the NorthernMax<sup>®</sup> Kit and suggested conditions.

Panel C - As in Panel B, but hybridized with a Cyclophilin RNA probe





### Competimer™ technology makes it feasible to use rRNA 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. 18S rRNA Primers and Competimers are supplied separately as 5 µM stocks of forward and reverse primers mixed in a 1:1 ratio. The 18S Competimers are modified at their 3' ends to block extension by DNA polymerase. By mixing 18S Primers with increasing amounts of 18S Competimers, the overall PCR amplification efficiency of 18S cDNA can be reduced without the primers becoming limiting and without loss of relative quantitation.

### 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	18S PCR Primer Pair † (5 µM)	-20°C
400 µL	18S PCR Competimers™† (5 µM) Modified oligonucleotides used in combination with 18S Primers. Allows 18S ribosomal 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‡

† Three different 18S Primers and Competimers are available, the following chart shows the size of the RT-PCR product produced from each of these primer pairs:

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

Part Number	Product name	Product size
AM1716	Classic 18S Internal Standards	489 bp
AM1717	Classic II 18S Internal Standards	324 bp
AM1718	Universal 18S Internal Standards	315 bp

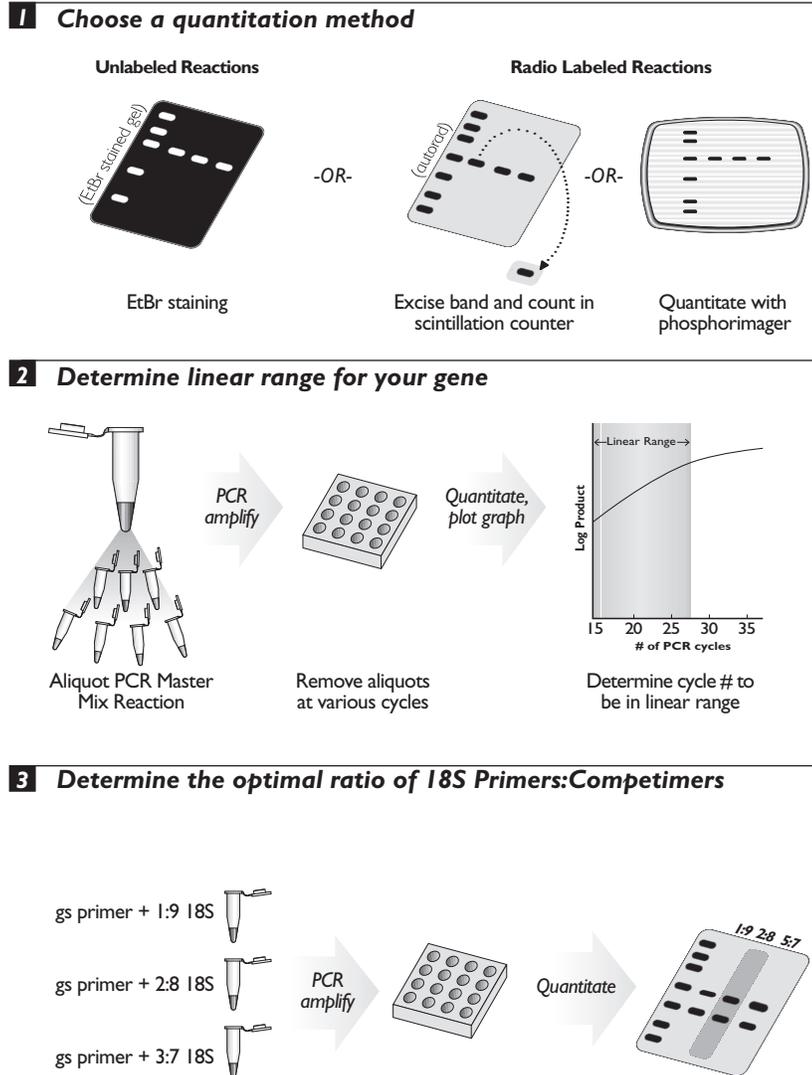
The 18S Primers and Competimers™ supplied with the kit are calibrated as a pair to generate a consistent RT-PCR signal. Pairs of 18S Primers and Competimers will have the same lot number. It is not advisable to mix 18S Competimers with 18S Primers from a different pair.

# Experimental Design

## 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 .

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. Furthermore, since 18S rRNA will be used as an internal standard, poly(A) RNA selection is not advisable.

### 2. RT primers

Usually it makes little or no difference whether random hexamers or decamers, or oligo dT is used as primer in a reverse transcription reaction. ***In order to use the 18S internal control, however, random primers must be used in the RT reaction.*** When ordering primers for reverse transcription, Life Technologies 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. Life Technologies gene specific primers meet all of the above criteria and have been functionally tested with QuantumRNA 18S Primers in multiplex RT-PCR.

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 18S Primers. Life Technologies has done multiplex RT-PCR using 18S 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.

## Quantifying PCR Products

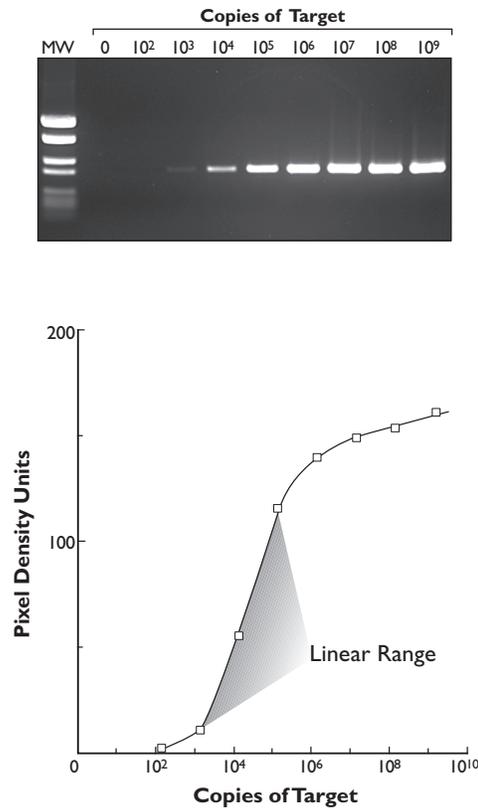
### A method to detect PCR products:

- 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 on page 12). 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  $\lambda$ Hind 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 [ $^{32}\text{P}$ ] dNTP

There are two ways to label a PCR with  $^{32}\text{P}$ . The PCR primers can be end labeled with [ $\gamma$ - $^{32}\text{P}$ ] ATP using T4 polynucleotide kinase, or [ $\alpha$ - $^{32}\text{P}$ ] 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  $\mu\text{L}$ , 10  $\mu\text{Ci}$  of 3000 Ci/mM) of [ $\alpha$ - $^{32}\text{P}$ ] 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  $6.5 \times 10^4$ ).

**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, digoxigenin, 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.



## 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

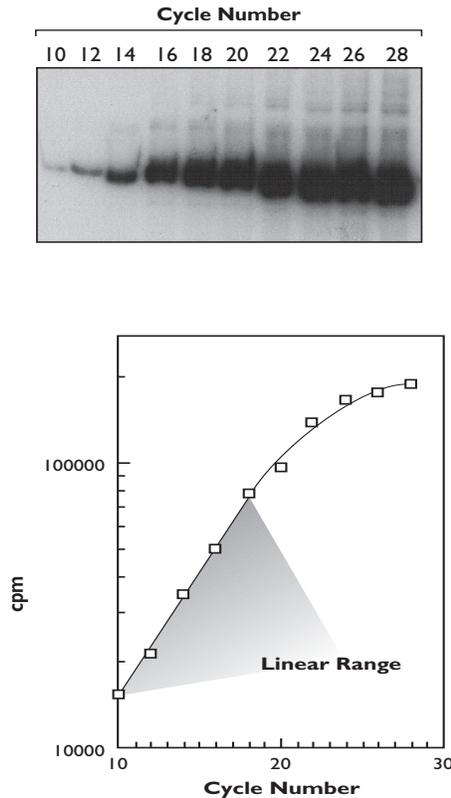
Reaction products accumulate during a PCR at a rate dependent on the amplification efficiency. The linear range of the reaction is defined as the period of the PCR in which the amplification efficiency is at its maximum and remains constant over a number of cycles. In other words, graphing “cycles vs. 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 on page 15, 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  $\mu\text{L}$  [ $\alpha\text{-}^{32}\text{P}$ ]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.

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

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

Amount	Component
up to 2.5 µg <sup>†</sup>	total RNA
4 µL	dNTP mix: 2.5 mM each
2 µL	random primers <sup>‡</sup> (50 µM)
to 16 µL	Nuclease-free Water

<sup>†</sup> much less RNA can be used if the target is abundant or if RNA is limiting.

<sup>‡</sup> 0.2–5 µM or 20–300 ng random primers (6–10mers) per reaction. Concentrations of first-strand primers within this range typically work equally well in reverse transcription.

- 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 <sup>†</sup>
1 µL	(optional) RNase inhibitor
100 units	M-MuLV RT

<sup>†</sup> 100 mM Tris-Cl pH 8.3, 500 mM KCL, 15 mM MgCl<sub>2</sub>

- 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 step 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-32P] dCTP (10 µCi/µL)

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

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**IMPORTANT!** Life Technologies 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. <sup>†</sup>	30 sec
72°C	30 sec

<sup>†</sup> Use the annealing temperature that works well for PCR of your gene of interest, the 18S Primers work well with annealing temperatures from 55–68°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® thermal cycler, 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 [Appendix B, “Electrophoresis Recipes, Set up, and Conditions”](#) on page 29. 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 phosphorimager, 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.

## Determine the Optimal Ratio of 18S Primers: Competimers

Since they compete for available resources, it is critical that the control target (18S) 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 18S control target amplify to give similar yields of product.

*A 3:7 ratio of 18S Primers to Competimers is appropriate for most genes if the experiment will have a relatively large dynamic range.* A 2:8 or lower ratio may be needed for extremely rare messages, and a 4:6 ratio may work best for relatively abundant messages. If less than a 1:9 ratio is needed, Life Technologies recommends doubling the amount of 18S Primers and Competimers in the reaction (i.e. use 8 µL of the Primer:Competimer mixture in step 2., “Set up the PCR cocktail” on page 19 instead of 4 µL). This is necessary to maintain a sufficient concentration of 18S 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 18S 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 3:7 ratio yields only 18S product, the following experiment is suggested to pinpoint the ideal 18S 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 18S 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 18S Primers and Competimers™ supplied with this kit are calibrated as a pair to generate a consistent RT-PCR signal. Pairs of 18S Primers and Competimers will have the same lot number. It is not advisable to mix 18S Competimers with 18S Primers from a different pair, because if you do, you may need to repeat the experiment that determines which ratio of 18S Primer:Competimer is best in your system.

---

1. Prepare 18S Primer: Competimer™ mixtures

Generally, messages with the following abundance levels require the 18S Primer:Competimer ratios shown:

Transcript Abundance	18S Primer: Competimer Ratio
Abundant transcripts	4:6
Moderately expressed transcripts	3:7
Rare transcripts	2:8
Extremely rare transcripts	1:9



Prepare the Primer:Competimer mixtures as follows:

18S Primer:Competimer ratio			
1:9	2:8	3:7	
9 $\mu$ L	8 $\mu$ L	7 $\mu$ L	18S Competimers
1 $\mu$ L	2 $\mu$ L	3 $\mu$ L	18S PCR Primer Pair

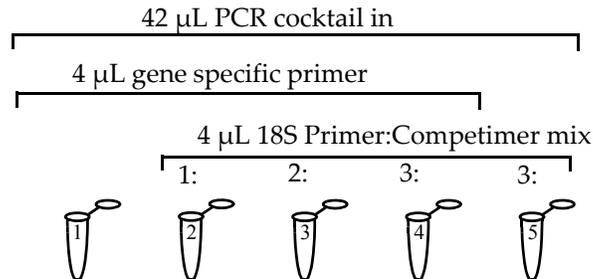
2. Set up the PCR cocktail

On ice: using an aliquot of an RT reaction performed as shown in step 4. (a.–d.) (on page 16), 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 $\mu$ L	template (RT reaction)
27.5 $\mu$ L	10X PCR Buffer
22 $\mu$ L	2.5 mM dNTPs
1.375 $\mu$ L	thermostable DNA polymerase
175 $\mu$ L	Nuclease-free Water
2.5 $\mu$ L	[ $\alpha$ -32P] dCTP

Aliquot 42  $\mu$ L of the PCR cocktail to 5 tubes labeled 1–5, and add gene specific primers and 18S PCR Primer and Competimer mixtures to the tubes as shown below:



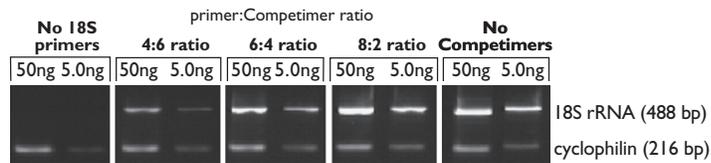
3. Perform the PCR

Use the temperatures and the number of cycles determined in the linear range determination experiment (section “[Determining the Linear Range for your RQ RT-PCR](#)” on page 14), and assess the results by gel electrophoresis.

4. Choose the best ratio

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

**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.



## Relative Quantitative RT-PCR

### Do Your Experiment

At this point, cycling parameters, linear range, and the optimal 18S 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 <sup>†</sup>	total RNA
4 µL	dNTP mix: 2.5 mM each
2 µL	random primers <sup>‡</sup> (50 µM)
to 16 µL	Nuclease-free Water

<sup>†</sup> much less RNA can be used if the target is abundant or if RNA is limiting.

<sup>‡</sup> 0.2–5 µM or 20–300 ng random primers (6–10mers) per reaction.

Concentrations of first-strand primers within this range typically work equally well in reverse transcription.

2. Denature RNA secondary structure

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**IMPORTANT!** If the RNA has any Mg<sup>++</sup> in the buffer (for example, from DNase reaction buffer), omit the heating step or the RNA could be degraded.

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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 <sup>†</sup>
1 µL	(optional) RNase inhibitor
100 units	M-MuLV RT

<sup>†</sup> 100 mM Tris-Cl pH 8.3, 500 mM KCL, 15 mM MgCl<sub>2</sub>

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	18S Primer:Competimer mixture (at the optimal ratio determined in step 4. on page 19)
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-32P] 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

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**IMPORTANT!** Life Technologies 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.

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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 18S Primers work well with annealing temperatures from 55–68°C.

## 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 18S 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.

## Troubleshooting RT-PCR

### 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™ 18S 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 steps 4. (a.–e.) (on page 16) 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:

Amount	Component
22 µL	10X PCR buffer
17.6 µL	18S PCR Primer Pair
4.4 U	thermostable DNA polymerase
to 216 µL	Nuclease-free Water




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**IMPORTANT!** Life Technologies 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.

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**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® thermal cycler, 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 18S PCR amplification (without Competimer) is very robust. The 18S product should be seen in all four samples.

**Verify that your RNA is OK**

The 18S PCR 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 step 4. (a.–e.) (on page 16). Use 1 µL of the RT in PCR:

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**IMPORTANT!** Life Technologies 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.

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Amount	Component
1 µL	RT reaction
5 µL	10X PCR buffer
4 µL	2.5 mM dNTPs
4 µL	18S PCR 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® thermal cycler, will probably require 1 minute at each temperature in the cycle for good results.



Run 10  $\mu$ L of the PCR on a 2% agarose gel stained with ethidium bromide. If the correct 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.

## 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. Occasionally, a processed (introns spliced out) pseudogene which yields the correct size fragments is amplified from genomic DNA.

### 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 (DNA-free™ Part no. 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 3' untranslated region).

### Unexpected primer interactions

In multiplex PCR there is always the possibility of unpredicted interaction between 18S and gene specific primers. This may be manifested as unexpected bands, specific to PCRs in which both primer pairs are present. Life Technologies has tested the 18S Primers in multiplex RT-PCR with over 100 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 18S. We suggest increasing the annealing temperature by two degree intervals and assessing the products after 30–35 cycles on an EtBr stained agarose gel.



## 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  $3.65 \times 10^5$  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).





# Materials Not Included with the Kit

## Required Materials not Included in the Kit

### RT-PCR reagents:

- Thermostable DNA polymerase (recommended: SuperTaq™, recombinant thermostable DNA polymerase, Part nos. AM2050, AM2052)
- Reagents for first strand cDNA synthesis (recommended: RETROScript® Kit Part no. AM1710, or Cells-to-cDNA™ II Part no. AM1722).

Reagents needed include random 6- to 10-mers, reverse transcriptase, and reaction buffer.

- Thermal cycler

### 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

## Related Products Available from Life Technologies

SuperTaq™ polymerase Part nos. AM2050, AM2052	Thermostable DNA Polymerase (includes 10X buffers and dNTPs)
RETROscript® Kit Part no. AM1710	First strand cDNA synthesis kit for RT-PCR. When purchased with SuperTaq™ polymerase, 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 Part no. AM1722, AM1723	The Cells-to-cDNA II Kit 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 Part no. AM1906	DNase treatment and removal reagents. This product contains Life Technologies'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	Life Technologies RNase-free tubes and tips are available in most commonly used sizes and styles. They are guaranteed RNase- and DNase-free.
Electrophoresis Reagents	Life Technologies offers gel loading solutions, agaroses, acrylamide solutions, powdered gel buffer mixes, nuclease-free water, and RNA and DNA molecular weight markers for electrophoresis.



# B

## Electrophoresis Recipes, Set up, and Conditions

### 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 <sub>2</sub> O

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

120 µL	10% ammonium persulfate in dH <sub>2</sub> O
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.

1. 1–2X denaturing gel loading buffer

This gel loading buffer is also available from Life Technologies; it is called NorthernMax<sup>®</sup> Gel Loading Buffer II, Part nos. AM8546G, AM8547

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

2. 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 <sub>2</sub> O

Stir at room temp, then add:

120 µL	10% ammonium persulfate in dH <sub>2</sub> O
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.

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**IMPORTANT!** Do not heat the samples before loading.

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#### 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.

3. 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, Life Technologies offers an all-purpose Gel Loading Solution for native gels, Part no. AM8556; this 10X solution is rigorously tested for nuclease contamination and functionality.



**WARNING! GENERAL SAFETY.** Using this product in a manner not specified in the user documentation may result in personal injury or damage to the instrument or device. Ensure that anyone using this product has received instructions in general safety practices for laboratories and the safety information provided in this document.

- Before using an instrument or device, read and understand the safety information provided in the user documentation provided by the manufacturer of the instrument or device.
  - Before handling chemicals, read and understand all applicable Safety Data Sheets (SDSs) and use appropriate personal protective equipment (gloves, gowns, eye protection, etc). To obtain SDSs, see the “Documentation and Support” section in this document.
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## Chemical safety



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**WARNING! GENERAL CHEMICAL HANDLING.** To minimize hazards, ensure laboratory personnel read and practice the general safety guidelines for chemical usage, storage, and waste provided below, and consult the relevant SDS for specific precautions and instructions:

Read and understand the Safety Data Sheets (SDSs) provided by the chemical manufacturer before you store, handle, or work with any chemicals or hazardous materials. To obtain SDSs, see the “Documentation and Support” section in this document.

- Minimize contact with chemicals. Wear appropriate personal protective equipment when handling chemicals (for example, safety glasses, gloves, or protective clothing).
- Minimize the inhalation of chemicals. Do not leave chemical containers open. Use only with adequate ventilation (for example, fume hood).
- Check regularly for chemical leaks or spills. If a leak or spill occurs, follow the manufacturer's cleanup procedures as recommended in the SDS.
- Handle chemical wastes in a fume hood.
- Ensure use of primary and secondary waste containers. (A primary waste container holds the immediate waste. A secondary container contains spills or leaks from the primary container. Both containers must be compatible with the waste material and meet federal, state, and local requirements for container storage.).
- After emptying a waste container, seal it with the cap provided.
- Characterize (by analysis if necessary) the waste generated by the particular applications, reagents, and substrates used in your laboratory.
- Ensure that the waste is stored, transferred, transported, and disposed of according to all local, state/provincial, and/or national regulations.
- **IMPORTANT!** Radioactive or biohazardous materials may require special handling, and disposal limitations may apply.

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## Biological hazard safety



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**WARNING! Potential Biohazard.** Depending on the samples used on this instrument, the surface may be considered a biohazard. Use appropriate decontamination methods when working with biohazards.



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**WARNING! BIOHAZARD.** Biological samples such as tissues, body fluids, infectious agents, and blood of humans and other animals have the potential to transmit infectious diseases. Follow all applicable local, state/provincial, and/or national regulations. Wear appropriate protective equipment, which includes but is not limited to: protective eyewear, face shield, clothing/lab coat, and gloves. All work should be conducted in properly equipped facilities using the appropriate safety equipment (for example, physical containment devices). Individuals should be trained according to applicable regulatory and company/institution requirements before working with potentially infectious materials. Read and follow the applicable guidelines and/or regulatory requirements in the following:



In the U.S.:

- U.S. Department of Health and Human Services guidelines published in Biosafety in Microbiological and Biomedical Laboratories found at: [www.cdc.gov/biosafety](http://www.cdc.gov/biosafety)
- Occupational Safety and Health Standards, Bloodborne Pathogens (29 CFR§1910.1030), found at: [www.access.gpo.gov/nara/cfr/waisidx\\_01/29cfr1910a\\_01.html](http://www.access.gpo.gov/nara/cfr/waisidx_01/29cfr1910a_01.html)
- Your company's/institution's Biosafety Program protocols for working with/handling potentially infectious materials.
- Additional information about biohazard guidelines is available at: [www.cdc.gov](http://www.cdc.gov)

In the EU:

- Check local guidelines and legislation on biohazard and biosafety precaution and refer to the best practices published in the World Health Organization (WHO) Laboratory Biosafety Manual, third edition, found at: [www.who.int/csr/resources/publications/biosafety/WHO\\_CDS\\_CSR\\_LYO\\_2004\\_11/en/](http://www.who.int/csr/resources/publications/biosafety/WHO_CDS_CSR_LYO_2004_11/en/)
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# Documentation and Support

## Obtaining SDSs

Safety Data Sheets (SDSs) are available from [www.lifetechnologies.com/sds](http://www.lifetechnologies.com/sds)

**Note:** For the SDSs of chemicals not distributed by Life Technologies, contact the chemical manufacturer.

## Obtaining support

For the latest services and support information for all locations, go to:

[www.lifetechnologies.com](http://www.lifetechnologies.com)

At the website, you can:

- Access worldwide telephone and fax numbers to contact Technical Support and Sales facilities
- Search through frequently asked questions (FAQs)
- Submit a question directly to Technical Support
- Search for user documents, SDSs, vector maps and sequences, application notes, formulations, handbooks, certificates of analysis, citations, and other product support documents
- Obtain information about customer training
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