MessageSensor™ RT Kit

High Sensitivity One Step RT-PCR

Part Number AM1745

MessageSensor™ RT Kit

(Part Number AM1745)

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I. Introduction

A. Background

The MessageSensor™ RT Kit for high sensitivity one-step real-time RT-PCR enables the synthesis of cDNA from total RNA, poly(A) RNA, or from a purified RNA transcript for use in PCR. When used with a suitable Taq polymerase, such as Ambion SuperTaq™ (P/N AM2050 and AM2052), the kit is capable of detecting specific, even rare, mRNA transcripts from the equivalent of a single mammalian cell (~10 pg total RNA) in a single-tube procedure.

The MessageSensor RT Kit includes an optimized set of reagents for exceptional sensitivity, and employs a convenient, user-friendly protocol. The kit is designed for flexibility; the cDNA can be used in real-time PCR* using fluorogenic probes, such as TaqMan®, or fluorescent dyes such as SYBR® Green I; or it can be used for end-point RT-PCR analyzed by gel electrophoresis. The MessageSensor RT technology can be readily adapted to high throughput, so that hundreds of samples can be evaluated in just a few hours. (Contact Ambion for information about bulk orders or custom kits: custom@ambion.com.)

Designed for quantitative RT-PCR

Real-time quantitative RT-PCR (qRT-PCR) is a powerful tool for measuring the abundance of mRNA transcripts in a cell. Effective qRT-PCR requires the reliable detection of targets over a broad range of input RNA. Figure 1 illustrates the ability of the MessageSensor RT Kit to quantify human glyceraldehyde-3-phosphate dehydrogenase (hGAPDH) with a one million fold range of input total RNA: 0.5 µg to 500 fg (r^2=0.998, triplicate samples). In the experiment shown, RT-PCR products were detected and quantified using a TaqMan probe, but similar results can be obtained using SYBR Green I detection. Unlike some kits that require different enzymes to reverse transcribe low and high amounts of input RNA, the MessageSensor RT Kit provides a "one size fits all" solution for even the most demanding qRT-PCR applications.

RNase H for optimal sensitivity

It has been reported that the action of RNase H after cDNA synthesis can improve the sensitivity of PCR detection (Polumuri et al. 2002). It is thought that if the RNA template is not degraded by RNase H following reverse transcription, it can bind to the newly synthesized cDNA

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and limit its accessibility during subsequent PCR amplification. Polumuri and colleagues (2002) tested the effects of RNase H treatment on RT-PCR detection sensitivity using an RNase H-minus RT to amplify three different target genes. Of these three targets, one was detected much more readily when an RNase H treatment step was included after reverse transcription. Experiments performed at Ambion show that the benefit of removing the RNA template after cDNA synthesis can be realized by either using an RNase H-plus RT for reverse transcription, or by synthesizing cDNA with an RNase H-minus RT and then treating the cDNA with RNase H to degrade the RNA template. These results strengthen the general finding that RNase H treatment is desirable for the most sensitive detection of RT-PCR products. For this reason the MessageSensor RT includes an RNase H-plus form of M-MLV RT so that a separate RNase H treatment step is not necessary.

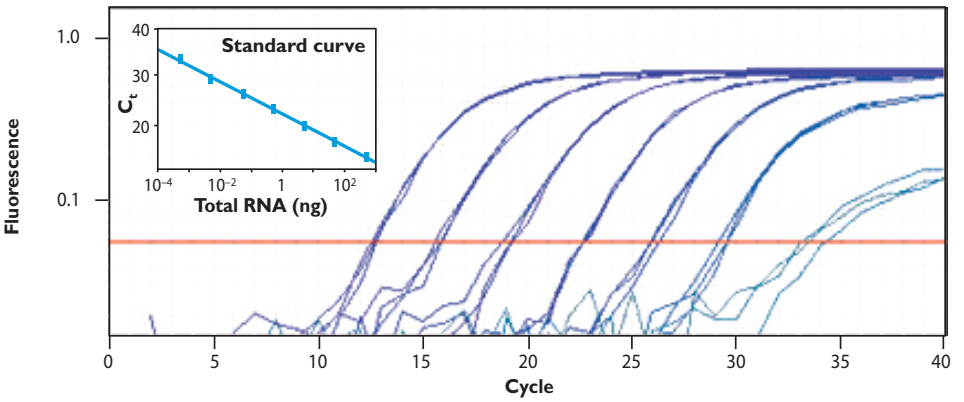

Figure 1. One-Step Real-time RT-PCR of hGAPDH from a One Million Fold Range of Input RNA Using the MessageSensor™ RT Kit and SuperTaq™ DNA Polymerase.

HeLa S3 total RNA (a 10 fold serial dilution from 500 ng to 500 fg) was used as template for RT-PCR using the MessageSensor RT Kit in conjunction with SuperTaq DNA Polymerase following the instructions in this booklet. Reactions included a TaqMan® probe, and were performed on an Applied Biosystems 7900HT Fast Real-Time PCR System. Triplicates of each input total RNA amount were analyzed (R^2=0.998, slope= –3.4). These results demonstrate the ability of the MessageSensor RT/SuperTaq system to generate reproducible data with great sensitivity over a broad range of input RNA; making it ideal for qRT-PCR.

RT at elevated temperatures

RNA commonly contains secondary structures at physiological temperatures. Even at 42°C, the most common temperature for reverse transcription, RNA can be highly structured. GC-rich sequences and secondary structures often present "roadblocks" that can prevent the translocation of RT enzymes along the RNA. In these cases, elevating the reaction temperature can "melt" these higher order structures and facilitate read-through by the RT (Malboeuf et al., 2001). In addition, higher temperatures can minimize nonspecific priming events, and improve the amplification of target cDNA for PCR detection. Some

reverse transcriptases, however, lose significant activity as the reaction temperature is increased more than a couple of degrees above 42°C. The M-MLV RT in the MessageSensor RT Kit, in contrast, maintains target sensitivity at reaction temperatures up to 50°C (Figure 2). Using the MessageSensor RT Kit, "difficult" RNA templates can be subjected to a range of RT reaction temperatures to obtain optimal results.

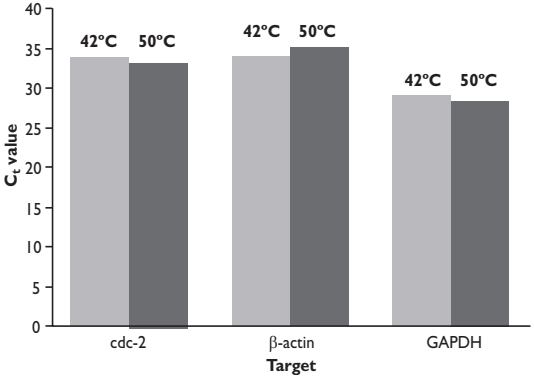

Figure 2. The M-MLV RT in the MessageSensor™ RT Kit Retains 100% Activity at 50°C.

Temperature comparison of one-step real-time qRT-PCR results using Ambion's total RNA from HeLa S3 cells. The total RNA (10 pg) was reverse transcribed at either 42°C or 50°C for 15 min, immediately followed by cDNA amplification. TaqMan® probes were used to detect the indicated targets. Reactions were performed on an Applied Biosystems 7900HT Fast Real-Time PCR System.

B. Materials Provided with the Kit and Storage Conditions

Reagents are provided for 50 RT-PCRs (25 µL each)

Amount	Component	Storage
1.75 mL	Nuclease-free Water	any temp*
500 µL	10X RT-PCR Buffer (the $MgCl_2$ concentration is 3 mM at 1X)	–20°C
200 µL	dNTP Mix (2.5 mM each dNTP)	–20°C
50 µL	RNase Inhibitor (10 U/µL)	–20°C
50 µL	M-MLV RT	–20°C
125 µL	10X Glycerol (8% glycerol for use with SYBR Green I)	–20°C
20 µL	hGAPDH Control Primers (10 µM each)	–20°C
100 µL	HeLa S3 Control RNA (10 ng/µL)	Below –70°C

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

C. Required Materials Not Provided with the Kit

For RT-PCR:

- RNA template
- Nuclease-free tubes and tips
- (optional) RNase*Zap*® Solution (P/N AM9780–AM9788), and DNA*Zap*™ Solution (P/N AM9890)
- Gene specific PCR primers for target(s) of interest
- Thermostable DNA Polymerase: This kit was designed and tested with Ambion SuperTaq™ DNA Polymerase (P/NAM2050 and AM2052), but the cDNA produced using this kit can be amplified with any thermostable DNA polymerase. See section *II.B.8. Thermostable DNA polymerases: recommendations and compatibility* on page 9 for more information.

For detection of RT-PCR products:

TaqMan® real-time detection

- TaqMan® probe
- ROX internal reference dye (250 µM solution)
- Thermal cycler capable of real-time reaction product detection, we used Applied Biosystems ABI PRISM® 7000, 7700, and 7900 Sequence Detection Systems, and the 7900HT Real-Time PCR System.

SYBR® Green I detection

- SYBR® Green I (Invitrogen)
- ROX internal reference
- Thermal cycler capable of real-time reaction product detection

For the positive control reaction, and for analysis by gel electrophoresis

- Material and equipment for native agarose gel analysis
- Thermal cycler

D. Related Products Available from Ambion

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.
DNA*Zap*™ Solution P/N AM9890	DNA degradation solution to avoid PCR contamination. This mixture is able to degrade high levels of contaminating DNA and RNA from surfaces instantly.
RNAqueous®-4PCR Kit P/N AM1914	The RNAqueous®-4PCR Kit provides RNA free of genomic DNA contamination from samples as small as 1 mg or 100 cells. The kit is especially suitable for RT-PCR applications and includes reagents to remove contaminating DNA from the isolated RNA.
RNase*Zap*® Solution P/N AM9780, AM9782, AM9784	RNase*Zap* RNase Decontamination Solution is simply sprayed, poured, or wiped onto surfaces to instantly inactivate RNases. Rinsing twice with distilled water will eliminate all traces of RNase and RNase*Zap* Solution.
*SuperTaq™ Polymerase P/N AM2050, AM2052	Thermostable DNA Polymerase (includes 10X buffers and dNTPs)
TURBO DNA-*free*™ Kit P/N AM1907	The TURBO DNA-*free* Kit employs Ambion's exclusive TURBO DNase (patent pending); a specifically engineered hyperactive DNase that exhibits up to 350% greater catalytic efficiency than wild type DNase I. The kit also includes a novel reagent for removing the DNase without the hassles or hazards of phenol extraction or alcohol precipitation—and without heat inactivation, which can cause RNA degradation. TURBO DNA-*free* is ideal for removing contaminating DNA from RNA preparations.

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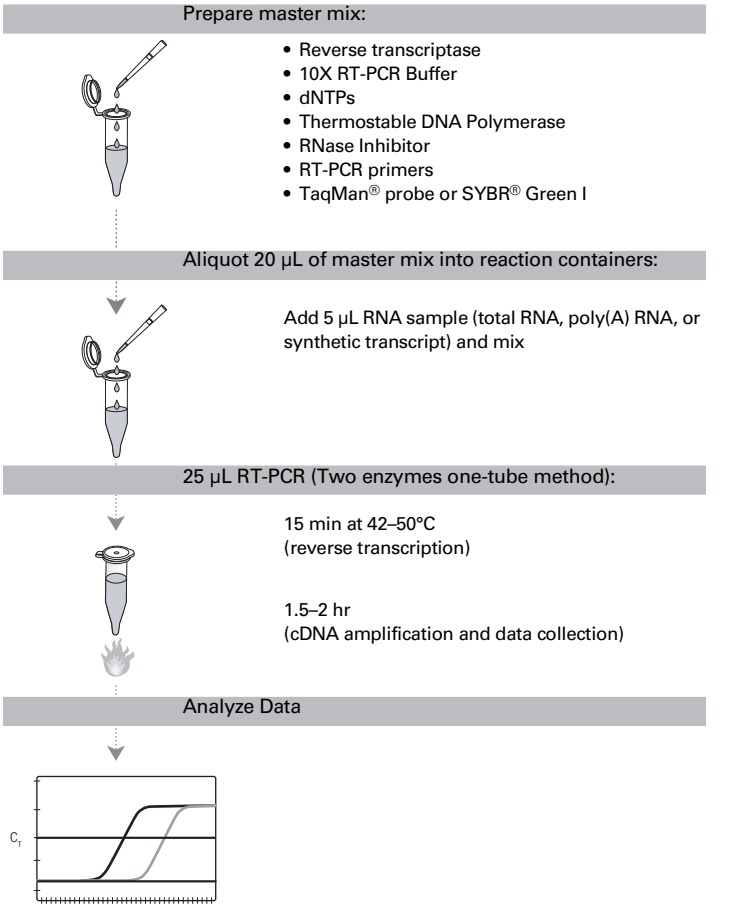
II. MessageSensor RT Procedure

A. Procedure Overview

Figure 3. Rapid One-Step Real-Time qRT-PCR with the MessageSensor™ RT Kit



B. Important Experimental Parameters

1. Negative Controls

a. Minus-RT control

Since most RNA preparations contain some contaminating genomic DNA, it is important to include a minus-reverse transcriptase (minus-RT) control in RT-PCR experiments. The minus-RT control will demonstrate that the RT-PCR product was amplified from cDNA, and not from genomic DNA. If a product is seen in the minus-RT control, it probably indicates that contaminating DNA is present in the sample. Typically, the minus-RT control is a mock reverse transcription containing all the RT-PCR reagents, except the reverse transcriptase.

b. Minus-template control

Another important control reaction is a minus-template control. The minus-template control includes all of the RT-PCR reagents except the RNA template; typically the RNA is simply substituted with nuclease-free water. No product should be synthesized in the minus-template control; if a product is amplified, it indicates that one or more of the RT-PCR reagents is contaminated with the amplicon.

2. Precautions to avoid DNA contamination

Material from previous PCR runs is the most serious potential source of DNA contamination. A good way to avoid contamination is to maintain separate areas of the lab for RT-PCR setup, and post-PCR sample analysis and RNA isolation (see O'Connell 2002, Chapter 2). It is also a good idea to keep different pipettors, tips, and reaction tubes in each area, stored in a dust-free environment. To avoid contaminating RT-PCR reagents, always use fresh pipet tips, and, ideally, a dedicated pipettor to dispense reagents. Consider aliquotting reagents to limit the number of pipetting events from stock solutions. Finally, as with most molecular biology procedures, handle reagents and pipettors with gloved hands, and change gloves frequently.

3. Use master mixes whenever possible

RT-PCR is a highly sensitive tool for analyzing RNA. Because the PCR greatly amplifies the target, errors are simultaneously amplified. Therefore, variability should be kept to a minimum whenever possible. When multiple reactions are assembled at the same time, prepare a cocktail or master mix of common reagents to minimize pipetting and contamination.

4. RNA purity

a. Genomic DNA contamination

Ambion typically recommends pre-treating RNA that will be analyzed by RT-PCR with DNase to remove genomic DNA. If PCR primers are designed to span an intron, however, DNase treatment may not be necessary. DNase requires a buffer with divalent cations for optimal activity, but heating RNA in a solution containing divalent cations may cause the RNA to degrade. The Ambion TURBO

DNA-*free*™ Kit (P/N AM1907) and DNA-*free*™ Kit (P/N AM1906) include both RNase-free Turbo DNase or DNase I, and a novel reagent to remove the DNase I and divalent cations from the RNA after the digestion reaction.

b. Other contaminants:

Many common methods for RNA purification use guanidinium thiocyanate and/or organic solvents such as phenol and chloroform. These compounds are protein denaturants and will inhibit the reverse transcriptase reaction if they are carried over to the RT-PCR along with the RNA. An alcohol precipitation step is necessary to remove such compounds.

Some RNA isolation procedures include a protease digestion step (such as Proteinase K). It is very important to thoroughly remove the protease to prevent digestion of the reverse transcriptase and/or the DNA polymerase. Phenol/chloroform extraction followed by alcohol precipitation will remove proteases from RNA.

5. RNA quality

The importance of using full-length RNA for reverse transcription depends on the application. Amplicons for real-time RT-PCR are typically short (70–250 bp). As a result, some degradation of the RNA may be tolerated, depending on the location of the primers. If it is not possible to obtain very high quality RNA, design primers to anneal to an internal region of the gene of interest. For truly quantitative RT-PCR, however, partially degraded RNA may not give an accurate representation of gene expression.

6. Primer and probe design

For the most efficient design of PCR primer and probe sets for real-time RT-PCR, we strongly recommend using primer design software. For TaqMan® real-time PCR, it is usually a good idea to design the TaqMan primer before choosing the PCR primers. Most primer design programs include adjustable parameters for optimal primer and probe design. These parameters consider primer/probe T_m, complementarity, and secondary structure as well as amplicon size and other important factors. Restricting the number of identical nucleotide runs is also recommended (see O'Connell 2002, Chapter 2 for further discussion). For best results choose an amplicon length of 70–100 bp. When designing amplicons in eukaryotic targets, choose PCR primers that span at least one intron in the target mRNA to prevent amplification of the target from contaminating genomic DNA.

7. RT-PCR primer and TaqMan® probe concentrations

TaqMan® real-time PCR

Gene-specific primer and probe concentrations may require adjustment depending on target abundance. An RT-PCR primer titration can identify the optimum primer concentration for a given target; typically this optimum is 100–500 nM for TaqMan® real-time PCR.

The optimal TaqMan probe concentration can also vary due to the target abundance; 80 nM is suggested as a general starting point for optimization. A probe titration of 50–100 nM can be conducted to pinpoint the best TaqMan probe concentration.

SYBR® Green I detection

For best results using SYBR® Green I detection, Ambion recommends that the RT-PCR primer concentrations be optimized for each target of interest. One common strategy for identifying the optimal primer concentration is to test several combinations of forward and reverse primer concentrations. For example, compare the real-time RT-PCR profiles for 50, 300, and 900 nM of both forward and reverse primers in a matrix analysis (thus, 9 combinations: 50 nM forward and 50 nM reverse, 50 nM forward and 300 nM reverse, 50 nM forward and 900 nM reverse, etc.). The goal of this matrix analysis is to pinpoint the primer concentrations that allow the T_m of each primer to be as closely matched as possible. The optimum primer concentration pair will be the one that yields the lowest C_T value while still minimizing the synthesis of non-specific products.

8. Thermostable DNA polymerases: recommendations and compatibility

The MessageSensor RT Kit was developed using Ambion SuperTaq™ Thermostable Taq DNA Polymerase (P/N AM2050 and AM2052). This formulation of SuperTaq is strongly recommended for optimal performance using the MessageSensor RT Kit. Other thermostable DNA Polymerases can also be used (Table 1); however, they have not been tested as extensively. The polymerases in Table 1 have been validated to very low levels of target detection (hGAPDH detection from 1 pg of total RNA) to ensure that sensitivity is not compromised. Before selecting an alternative PCR polymerase, consider the following factors:

- Real-time PCR using TaqMan probes requires a DNA polymerase that retains 5' exonuclease activity to displace and hydrolyze the TaqMan probe (Bustin 2002 and Zhao 2002). Titanium Taq DNA Polymerase, for example, lacks 5' exonuclease activity; thus this enzyme is incompatible with TaqMan detection.
- Some thermostable DNA Polymerases are incompatible with the 10X RT-PCR Buffer provided with the MessageSensor RT Kit. For example, Applied Biosystems AmpliTaq Gold® DNA Polymerase should be used with the buffer supplied by the manufacturer, adjusted to 3 mM final $MgCl_2$ concentration, to support TaqMan RT-PCR.

Table 1. DNA Polymerases Tested with the MessageSensor™ RT Kit and Recommended Reaction Buffers

DNA polymerase	Vendor	Compatibility with MessageSensor RT	Buffer Compatibility	
			10X RT-PCR Buffer supplied with the MessageSensor RT Kit	Buffer supplied with enzyme
SuperTaq™	Ambion	Strongly Recommended	Yes	n/a
AmpliTaq Gold®	Applied Biosystems	Yes (R^2>0.998)	No	Yes, but adjust final $MgCl_2$ conc to 3 mM
JumpStart Taq	Sigma-Aldrich	Yes (R^2>0.999)	Yes	Yes, but adjust final $MgCl_2$ conc to 3 mM
Platinum Taq	Invitrogen	Yes (R^2>0.998)	Yes	Yes, but adjust final $MgCl_2$ conc to 3 mM
TaKaRa Ex Taq for RT-PCR	TaKaRa/ Panvera	Yes (R^2>0.999)	Yes	Yes, but adjust final $MgCl_2$ conc to 3 mM
Taq DNA Polymerase in Storage Buffer B	Promega	Yes (R^2>0.999)	Yes	Yes, but adjust final $MgCl_2$ conc to 3 mM

C. One-Step RT-PCR Procedures

In the next three sections, one-step RT-PCR procedures are presented for three different methods of detecting the reaction products:

RT-PCR product detection	Instructions begin on:
Real-time RT-PCR with TaqMan® Probe Detection	page 10
Real-time RT-PCR with SYBR® Green I Detection	page 14
End-point One-Step RT-PCR	page 17
Two-Step RT-PCR Procedure	page 23

D.I. Real-time RT-PCR with TaqMan® Probe Detection

1. Prepare working concentrations of RT-PCR primers and TaqMan® probe

- Prepare a gene specific primer mixture containing 10 µM of each PCR primer (forward and reverse) in 10 mM Tris-HCl pH 8, 0.1 mM EDTA, or in nuclease-free water.
- Dilute TaqMan® probe to a working concentration of 2 µM in nuclease-free water. Minimize exposure of the probe to light.

2. Plan the reaction setup

qRT-PCR experiments using TaqMan probes must include samples to be used for quantification and verification of the results. At a minimum, include duplicates of each reaction (triplicates are even better). Two of the common quantification methods are the following:

- Include a standard curve: make a standard curve by using 10-fold serial dilutions of input RNA in the RT-PCR. A range of 1000-fold (or greater) may be necessary depending on the target abundance. Also, the minimum and maximum RNA amounts should extend above and below the limit of detection to help differentiate between specific and non-specific products. The cycle thresholds (C_T) of experimental samples can then be compared with the standard curve to quantify the target mRNA levels.
- Use the $\Delta\Delta C_T$ method of quantitation: this approach provides a measurement of differences in the levels of an endogenous control gene relative to a target gene without the need for a standard curve.

Detailed discussion of these techniques is beyond the scope of this protocol; Bustin 2002, Freeman et al. 1999, and O'Connell 2002 are good sources of information on this topic.

3. Assemble the reaction

a. Thaw all reaction components on ice, except the M-MLV RT and DNA polymerase which should be kept at –20°C. Gently mix, then centrifuge components before opening.

b. In a nuclease-free tube on ice, prepare a master mix with all the RT-PCR reaction components ***except the M-MLV RT and the RNA template.***
The reaction setup shown below is for a single 25 µL reaction, prepare enough master mix for all of the samples in the experiment, and include 5% overage to compensate for pipetting error.
Mix well and centrifuge briefly.

Amount	Component
8.8 µL	Nuclease-free Water*
2.5 µL	10X RT-PCR Buffer
4 µL	dNTP Mix (2.5 mM each)
1 µL	Gene Specific Primer Mix (10 µM each)
1 µL	TaqMan® Probe (2 µM)
1 µL	RNase Inhibitor (10 U/µL)
0.5 µL	50X ROX internal reference (250 µM)
0.2 µL	SuperTaq™ (5 U/µL) (or 1 U thermostable DNA Pol; see section II.B.8 on page 9)
1 µL	M-MLV RT (for minus-RT control use Nuclease-free Water)
5 µL	RNA (1 pg to 1 µg)† (for minus-template control use Nuclease-free Water)

* This amount of water assumes that the RNA template volume will be 5 µL. Up to 10 µL of RNA template can be added to the reaction by reducing the volume of water accordingly.

† These values are a recommended starting range. In reality, the appropriate amount of input RNA will depend on the abundance of the target transcript, and the quality and type of RNA [total, poly(A), or synthetic].

c. Remove an aliquot of the master mix for the minus-RT controls to a nuclease-free tube, and add 1 µL of Nuclease-free Water per reaction (plus 5% overage) in place of the reverse transcriptase. Mix and briefly centrifuge.

d. To the remainder of the master mix, add 1 µL of M-MLV RT per reaction (plus 5% overage), place on ice. Gently mix and briefly centrifuge.

IMPORTANT

Close or cover the reaction containers to minimize contamination before and after reagents are added.

e. On ice, dispense 20 µL of master mix into the reaction containers appropriate for the real-time thermal cycler.

f. To each experimental sample and to the minus-RT controls, add 5 µL of RNA and mix.
To each minus-template control add 5 µL of Nuclease-free Water in place of the RNA template.

4. Use the following cycling parameters:

	Stage	Reps	Temp	Time
Reverse transcription	1	1	42–50°C	15 min
RT inactivation/Taq activation	2	1	95°C	5 min
Amplification	3	40	95°C	15 sec
			60°C	40–60 sec

NOTE

Cycling parameters are for the Applied Biosystems ABI Prism® 7000, 7700, and 7900 Sequence Detection Systems, and the 7900HT Real-Time PCR System. For other instruments refer to the operating manual for cycling instructions.

5. Collect data and analyze results

Negative controls

There should be no visible RT-PCR products from either the minus-RT or the minus-template negative controls. If amplification occurs in these controls see section *III.C. RT-PCR Products in the Negative Control Reaction(s)* on page 21.

Experimental reactions

The exponential nature of PCR amplification means that the amount of product theoretically doubles after each cycle. This doubling can be mathematically described as follows:

$$P = P_0 \times 2^n$$

where P = amount of product at cycle n

P_0 = amount of product at the start of reaction

n = number of PCR cycles

In TaqMan real-time PCR, the data are represented by two major parameters: fluorescence intensity and cycle threshold (C_T). Fluorescence intensity of the TaqMan probe is recorded during the data collection phase of the PCR. The C_T is the minimum cycle number at which the fluorescent signal can be statistically differentiated from the background. The C_T always occurs during the exponential phase of the reaction where the equation above holds true. Typically the fluorescence intensity is plotted against the C_T to create a standard curve that can be used to identify the unknown sample concentration. When the efficiency of amplification is 100%, the slope of this standard curve is –3.3. However, a number of variables can affect the efficiency of the PCR, resulting in a slope of less than –3.3. The efficiency (Eff) of the reaction can be calculated as follows:

$$Eff = 10^{(-1/slope)} - 1$$

Thus a slope of –3.9 would correspond to 80% efficiency.

The quality of the data from a real-time RT-PCR using TaqMan detection can be evaluated using several criteria.

- For any standard curve, the correlation coefficient (R^2) should be close to 1.0 as possible; for example $R^2 > 0.98$.
- Second, the efficiency of the PCR should be 80–100% ($-3.9 \geq slope \geq -3.3$), although valid data can be obtained that fall outside of this range.
- The precision of the data across replicates is a good measure of the data quality. At Ambion, we often observe ≤ 0.5 C_T deviation among replicates, even at input RNA levels that are near the limit of detection.

The goal of real-time RT-PCR experiments is often to quantify the amount of a particular transcript. Some experiments strive for absolute quantification in order to calculate the actual number of copies of an amplicon. For absolute quantification, an absolute standard, such as a purified transcript of a known concentration, is required for reference. In other cases, relative quantification may be desirable, for example, comparing differential expression of a gene plus or minus some treatment

state. For both cases, quantification is accomplished by comparing C_T values, where a 1 C_T shift corresponds to a 2-fold difference in product accumulation; see the review by Bustin, 2000, for more information.

D.II. One Step Real-time RT-PCR with SYBR® Green I Detection

1. Prepare a stock solution of RT-PCR primers

Real-time RT-PCR analysis using SYBR® Green I detection often requires optimizing the concentration of forward and reverse RT-PCR primers (see section II.B.7 on page 8). For optimization experiment(s), Ambion recommends preparing 10 µM stock solutions of each RT-PCR primer, and diluting these stocks as needed in 10 mM Tris-HCl pH 8, 0.1 mM EDTA, or in nuclease-free water. Once the optimal forward and reverse RT-PCR primer concentrations have been identified, prepare a 10X solution containing both primers for use in the reaction described in step 4 on page 14.

2. Plan the reaction setup

Include duplicates (or triplicates) of each reaction. As for TaqMan® detection, also consider including samples to be used for quantitation and verification of the results as described in step C.2 on page 10. See step 6 on page 16 or Lekanne Deprez 2002 for further information on analysis of SYBR Green I in real-time RT-PCR.

3. Add SYBR® Green I to the 10X RT-PCR Buffer

a. Dilute SYBR Green I 100-fold: add 2 µL SYBR Green I to 198 µL Nuclease-free Water in a nuclease-free tube. Mix thoroughly.

b. Add 12.5 µL of diluted SYBR Green I to 487.5 µL 10X RT-PCR Buffer and mix thoroughly.
When the 10X RT-PCR Buffer is used in a 25 µL RT-PCR, the SYBR Green I will have a final dilution of 1:40,000.

4. Assemble the reaction:

a. Thaw all reaction components on ice, except the M-MLV RT and DNA polymerase which should be kept at –20°C. Gently mix, then centrifuge components before opening.

b. In a nuclease-free tube on ice, prepare a master mix with all the RT-PCR reagents ***except M-MLV RT and RNA template***.
The reaction setup below is for a single 25 µL reaction, prepare enough master mix for all samples in the experiment, including ~5% overage to compensate for pipetting error.

Mix well and centrifuge briefly.

Amount	Component
5.8 µL	Nuclease-free Water*
2.5 µL	10X RT-PCR Buffer + SYBR Green I (from step 3)
2.5 µL	10X Glycerol
4 µL	dNTP Mix (2.5 mM each)
1 µL	RNase Inhibitor (10 U/µL)
0.5 µL	50X ROX internal reference (250 µM)
0.2 µL	SuperTaq (5 U/µL) (or 1 U thermostable DNA Pol; see section II.B.8 on page 9)
2.5 µL	10X gene specific RT-PCR primer mix†
1 µL	M-MLV RT (for minus-RT control use Nuclease-free Water)
5 µL	RNA (1 pg to 1 µg)‡ (for minus-template control use Nuclease-free Water)

* This amount of water assumes that the RNA template volume will be 5 µL. Up to 10 µL of RNA template can be added to the reaction by reducing the volume of water accordingly.

† The concentration of the forward and reverse RT-PCR primers in the 10X stock solution should be based on a primer optimization experiment as described in section II.B.7 *SYBR® Green I detection* on page 9.

‡ These values are a recommended starting range. In reality, the appropriate amount of input RNA will depend on the abundance of the target transcript, and the quality and type of RNA [total, poly(A), or synthetic].

c. Transfer master mix for the minus-RT controls to a nuclease-free tube, and add 1 µL of Nuclease-free Water per reaction (plus 5% overage) in place of the M-MLV RT. Mix and briefly centrifuge.

d. Add 1 µL of M-MLV RT per reaction (plus 5% overage) to the rest of the master mix and place on ice. Gently mix and briefly centrifuge.

IMPORTANT

Close or cover the reaction containers to minimize contamination before and after reagents are added.

e. On ice, dispense 20 µL of master mix into the reaction containers appropriate for the real-time thermal cycler.

f. To each experimental sample and to the minus-RT controls, add 5 µL of RNA and mix.
To each minus-template control add 5 µL of Nuclease-free Water in place of the RNA template.

5. Use the following cycling parameters:

	Stage	Reps	Temp	Time
Reverse transcription	1	1	42°C	15 min
RT inactivation/Taq activation	2	1	95°C	5 min
Amplification	3	40	95°C	15 sec
			60°C	30 sec
Detection Step	4	1	*76–80°C	40–60 sec
Dissociation Curve	5	—	default parameters	

* The detection step should be done at a temperature just below the T_m of the amplicon (but above the T_m of any non-specific products) to avoid fluorescent detection of the non-specific product. At this temperature, the duplexed specific product is readily detected, but non-duplexed, non-specific products are not.

NOTE

Cycling parameters are for the Applied Biosystems ABI Prism® 7000, 7700, and 7900 Sequence Detection Systems, and the 7900HT Real-Time PCR System. For other instruments refer to the operating manual for cycling instructions.

6. Collect data and analyze results

SYBR Green I fluorescence

On binding the minor groove of duplex DNA, SYBR Green I fluorescence increases roughly 100-fold. Since it binds indiscriminately to all double-stranded DNA, however, all duplex DNA products formed in the PCR will generate a signal in the presence of SYBR Green I. As a result, careful analysis of real-time RT-PCR data is necessary when SYBR Green I is used. The following strategy is recommended to determine whether the data quality is sufficient for gene expression studies (Lekanne Deprez et al. 2002).

Validate the data

First, conduct a dissociation (melting) curve of the products immediately following the RT-PCR run. Software included with current real-time PCR instruments often feature a default program for this purpose. Ideally, the experimental samples should yield a sharp peak (first derivative plot) at the melting temperature of the amplicon, whereas the minus-RT and minus-template negative controls will not generate significant fluorescent signal. This result indicates that the products are specific, and that SYBR Green I fluorescence is a direct measure of accumulation of the product of interest. It is also advisable to run the products on an agarose gel to confirm that a single band of the expected size is observed.

If there is more than one product visible on a gel, or if the dissociation curve reveals a series of peaks, it indicates that there is not enough discrimination between specific and non-specific reaction products. To obtain meaningful data, optimization of the RT-PCR would be necessary.

Further optimization

Optimization may require changing one or more of the following reaction parameters: primer and/or Mg^{2+} concentration, detection temperature, cycling parameters, etc. It has also been suggested that DTT may reduce the detection sensitivity of some targets when using SYBR Green I (Lekanne Deprez et al., 2002). In certain cases, the dissociation curve may indicate a more suitable data collection temperature.

D.III. End-point One-Step RT-PCR

This procedure is for one-step RT-PCR that will be analyzed by gel electrophoresis at the end of a set number of PCR cycles.

1. Prepare a 10 µM solution of RT-PCR primers

Prepare a gene specific primer mixture containing 10 µM of each RT-PCR primer (forward and reverse) in 10 mM Tris-HCl pH 8, 0.1 mM EDTA, or in nuclease-free water.

2. Assemble the reaction

a. Thaw all reaction components on ice, except the M-MLV RT and DNA polymerase which should be kept at –20°C. Gently mix, then centrifuge components before opening.

b. In a nuclease-free tube on ice, prepare a master mix with all the RT-PCR reagents ***except M-MLV RT and RNA template.***
The reaction setup shown below is for a single 25 µL reaction, prepare enough master mix for all of the samples in the experiment, and include 5% overage to compensate for pipetting error.

Mix well and centrifuge briefly.

Amount	Component
10.3 µL	Nuclease-free Water*
2.5 µL	10X RT-PCR Buffer
4 µL	dNTP Mix (2.5 mM each)
1 µL	Gene Specific Primer Mix (10 µM each)
1 µL	RNase Inhibitor (10 U/µL)
0.2 µL	SuperTaq (5 U/µL) (or 1 Uthermostable DNA Pol, see section II.B.8 on page 9)
1 µL	M-MLV RT (for minus-RT control use Nuclease-free Water)
5 µL	RNA (1 pg to 1 µg)† (for minus-template control use Nuclease-free Water)

* This amount of water assumes that the RNA will be in 5 µL. Up to 10 µL of RNA can be added to the reaction by adjusting the water volume accordingly.

† These values are a recommended starting range. In reality, the appropriate amount of input RNA will depend on the abundance of the target transcript, and the quality and type of RNA [total, poly(A), or synthetic].

c. Remove an aliquot of the master mix for the minus-RT controls to a nuclease-free tube, and add 1 µL of Nuclease-free Water per reaction (plus 5% overage) in place of the reverse transcriptase. Mix and briefly centrifuge.

d. To the remainder of the master mix, add 1 µL of M-MLV RT per reaction (plus 5% overage), place on ice. Gently mix and briefly centrifuge.

e. On ice, dispense 20 µL of master mix into the reaction containers appropriate for the thermal cycler.

IMPORTANT

Close or cover the reaction containers to minimize contamination before and after reagents are added.

f. To each experimental sample and to the minus-RT controls, add 5 µL of RNA and mix.
To each minus-template control add 5 µL of Nuclease-free Water in place of the RNA template.

3. Use the following cycling parameters:

	Stage	Reps	Temp	Time
Reverse transcription	1	1	42–50°C	15 min
RT inactivation/Taq activation	2	1	95°C	5 min
Amplification	3	40	95°C	15 sec
			annealing temp*	40 sec

* Use the annealing temp suggested by the primer design software, 60°C is often a good starting point for cycling temperature optimization.
An extension step may be required after the anneal/extension step if amplicon lengths are >0.5 kb.

4. Analysis of results

Separate the reaction products by agarose gel electrophoresis. Load as much of the 25 µL reaction as possible on the gel, and stain the DNA with ethidium bromide or other dsDNA dyes.

Negative controls

There should be no visible RT-PCR products from either the minus-RT or the minus-template negative controls. If amplification occurs in these controls see section *III.C. RT-PCR Products in the Negative Control Reaction(s)* on page 21 for troubleshooting suggestions.

Experimental reactions

Ideally there will be a single band of the expected size in the lanes with the experimental samples.

III. Troubleshooting

A. Using the Positive Control

The MessageSensor RT Kit includes HeLa S3 Control RNA and hGAPDH Control Primers which will amplify a ***226 bp DNA fragment*** using one-step RT-PCR. The positive control reaction product can be readily visualized on an ethidium bromide-stained agarose gel.

The purpose of the positive control reaction is to verify that the MessageSensor RT Kit is functioning properly.

The instructions below show the volumes necessary to conduct the two reactions (plus 5% overage) that make up the positive control: the positive control reaction itself, and a minus-template negative control.

Positive control reaction setup

a. Assemble the following master mix on ice in nuclease-free tubes:

Amount	Component
21.6 µL	Nuclease-free Water
5.3 µL	10X RT-PCR Buffer
8.4 µL	dNTP Mix (2.5 mM each)
2.1 µL	hGAPDH Control Primers (10 µM each)
2.1 µL	RNase Inhibitor (10 U/µL)
0.4 µL	SuperTaq (5 U/µL) (or 2 U thermostable DNA Pol, see section II.B.8 on page 9)
2.1 µL	M-MLV RT

b. Add HeLa S3 Control RNA or Nuclease-free Water as follows:

Minus-template	Positive control	Component
20 µL	20 µL	Master mix (from 4.a above)
– –	5 µL	HeLa S3 Control RNA (10 ng/µL)
5 µL	– –	Nuclease-free Water

Cycling parameters

	Stage	Reps	Temp	Time
Reverse transcription	1	1	42°C	15 min
RT inactivation/Taq activation	2	1	95°C	5 min
Amplification	3	40	95°C	15 sec
			60°C	40 sec

NOTE

Cycling parameters are for the Applied Biosystems ABI Prism® 7000, 7700, and 7900 Sequence Detection Systems, and the 7900HT Real-Time PCR System. For other instruments refer to the operating manual for cycling instructions.

Analysis of results

There should be no product amplified from the minus-template RT-PCR. This shows that the RT-PCR reagents are not contaminated with RNA, or with DNA containing the GAPDH amplicon.

The positive control reaction should yield a single 226 bp product that is readily visible on a 2% agarose/TBE gel stained with ethidium bromide. If the positive control does not show the expected result, there may be a problem with one of the RT-PCR reagents, the DNA polymerase, or the thermal cycler.

B. No RT-PCR Product or Unexpected RT-PCR Products

Do the positive control reaction

Consider doing the positive control reaction to verify that the kit is working as expected; instructions are in section III.A (previous).

Check the quality of the RNA sample

PCR primers targeting a sequence at the 5' or 3' end of an RNA fragment are especially vulnerable to the reduced cDNA yield resulting from reverse transcription of partially degraded RNA. Primers designed to target a more centrally located target sequence, however, can tolerate some degradation of the RNA template.

The overall quality of a total RNA preparation can be assessed by electrophoresis on a denaturing agarose gel stained with ethidium bromide or another nucleic acid dye. An aliquot of RNA markers and/or an aliquot of RNA known to be intact should be run as a positive control to rule out gel artifacts. In intact RNA, the ribosomal RNA (rRNA) bands will be fairly sharp and intense, and the larger rRNA band will be about twice as intense as the smaller band. A diffuse smear of mRNA may also be visible. Degradation of the RNA will be reflected by smearing of ribosomal RNA bands. DNA contamination, if it is present, will form a high molecular weight smear or band migrating above the larger rRNA band. These same criteria can be applied to analysis of RNA using an Agilent bioanalyzer.

If the sample RNA is degraded or partially degraded, then using higher quality RNA in your experiments will probably increase RT-PCR sensitivity and reproducibility.

The RT-PCR requires optimization

There are many factors that can influence RT-PCR; these are discussed in section *II.B. Important Experimental Parameters* on page 7. Probably the most common causes of failed, or low efficiency RT-PCRs are suboptimal primer sequence and annealing temperature.

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

Redesign the PCR primers.
Ambion strongly recommends that computer software be used to design PCR primers and TaqMan probes. TaqMan primer and probe sets are highly species specific and slight mismatches reduce optimum annealing, thereby reducing sensitivity of real-time RT-PCR.

Optimize the magnesium concentration.
Magnesium concentration can be very important in real-time RT-PCR using the TaqMan detection method. Generally a final $MgCl_2$ concentration of 3 mM is effective for most targets, thus the 10X RT-PCR Buffer included in the MessageSensor RT Kit provides 3 mM $MgCl_2$ final concentration. For some targets, it may be necessary to test a range of magnesium concentrations of up to 6 mM to optimize the RT-PCR.

RNA secondary structure

Persistent secondary structure in the RNA template may interfere with the reverse transcription reaction. Increasing the reaction temperature from 42°C to as high as 50°C may improve sensitivity of detection for some targets.

The mRNA is undetectable, or is not present in the sample

Use more RNA template
If less than 1 µg RNA was used in RT-PCR, and the mRNA is rarely expressed, the amount of input RNA can be increased above 1 µg. Alternatively, for targets that are polyadenylated, poly(A) selected RNA can be used instead of total RNA to increase the amount of target in the reaction.

C. RT-PCR Products in the Negative Control Reaction(s)

Contamination with DNA from previous PCRs

If RT-PCR products are produced in the minus-RT or minus-template control reaction, it is possible that the reaction was contaminated with DNA. The most common source of DNA contamination is amplicons from previous experiments. Segregating areas of the laboratory for PCR assembly and RT-PCR analysis, and minimizing contamination from environmental sources can help prevent such contamination. See section *II.B.2. Precautions to avoid DNA contamination* on page 7 for further details. Also, DNA*Zap*™ DNA Degradation Solution (P/N AM9890) can be applied to the bench top, pipettors, and other equipment to degrade potentially contaminating DNA. After treatment, the DNA*Zap* is easily removed by a quick rinse with nuclease-free water.

RNA contains genomic DNA contamination

Treat input RNA with DNase I
If the sample has not been treated with DNase I, consider treating a small volume of RNA sample to check for potential genomic DNA contamination. If the sample has been treated with DNase I, in some cases a second DNase I treatment will be beneficial.

Design PCR primers to span an intron

One common strategy for eliminating RT-PCR products from genomic DNA is to design primers that span an intron. In PCR, the primers will hybridize to an exon-exon interface which is present in mRNA, but not in genomic DNA.

The DNA polymerase exhibited reverse transcriptase activity

There is a report that thermostable DNA polymerases, including native Taq, can exhibit low-level intrinsic reverse transcriptase activity (Maudru and Peden 1997). This could potentially cause an RT-PCR product to be made from a minus-RT control in the absence of contaminating DNA.

IV. Appendix

A. Two Step RT-PCR Procedure

The MessageSensor RT Kit was developed with outstanding sensitivity and range of detection in a one-step, one-tube format. However, if desired, the RT-PCR can be performed in two sequential reactions.

Reverse transcription and primer choice

In the first step, the template RNA is reverse transcribed using random primers, oligo(dT), or gene specific primers. When the template is partially degraded, the final RT-PCR yield may be somewhat higher when the reverse transcription reaction is primed with random primers as opposed to oligo(dT) or gene-specific primers (see Innis et al. 1990 Chapter 3, and O'Connell 2002 Chapter 2 for further discussion). Amplicons located near the 5' end of the gene may be better primed with random primers rather than oligo(dT) since random primers hybridize at sites all along the mRNA. In contrast, oligo(dT) primers anneal to poly(A) tails, and are often used to prime cDNA synthesis when it is important for full-length, or at least the 3' region of RNA, to be represented in the cDNA. Gene specific primers will prime only from the target mRNA. Using gene specific primers may be useful if the mRNA of interest is at a low copy number (O'Connell 2002, Chapter 2).

PCR

After reverse transcription the cDNA is amplified by PCR with gene specific primers and a suitable thermostable DNA Polymerase. The result is analyzed by agarose gel electrophoresis.

1. Reverse transcription reaction

a. Defrost all reaction components on ice except the M-MLV RT, keep it at –20°C until just before use. Gently mix and centrifuge components before opening and close tubes when not in use.

b. In a nuclease-free tube on ice, prepare an RT master mix with all of the reagents ***except M-MLV RT and RNA template***.
The reaction setup shown below is designed for a single 20 µL reaction, prepare enough RT master mix for all of the samples in the experiment, including 5% overage to compensate for pipetting error.
Mix well and centrifuge briefly.

Using random primers or oligo(dT) primers	Using gene specific primers	Component
5 µL	6.5 µL	Nuclease-free Water*
2 µL	2 µL	10X RT-PCR Buffer
4 µL	4 µL	dNTP Mix (2.5 mM each)
2 µL	– –	random primers (50 µM) or oligo(dT) (50 µM)
– –	0.5 µL	gene specific primers (10 µM each)
1 µL	1 µL	RNase Inhibitor (10 U/µL)
1 µL	1 µL	M-MLV RT (for minus-RT control use Nuclease-free Water)
5 µL	5 µL	RNA (100 ng to 1 µg) (for minus-template control use Nuclease-free Water)

* This amount of water assumes that the RNA template volume will be 5 µL. Up to 10 µL of RNA template can be added to the reaction by adjusting the volume of water accordingly.

c. Remove an aliquot of the master mix for the minus-RT control to a nuclease-free tube, and add 1 µL of Nuclease-free Water per reaction in place of the reverse transcriptase. Mix and briefly centrifuge.

d. To the remainder of the master mix, add 1 µL of MMLV-RT per reaction (plus any overage volume), place on ice. Gently mix and briefly centrifuge.

IMPORTANT

Close or cover the reaction containers to minimize contamination before and after reagents are added.

e. On ice, dispense 15 µL of RT master mix into nuclease-free reaction containers.

f. To each experimental sample and to the minus-RT controls, add 5 µL of RNA and mix.
To each minus-template control add 5 µL of Nuclease-free Water in place of the RNA template.

g. Incubate at 42–50°C for 15–30 minutes. Then heat-denature the reverse transcriptase at 95°C for 10 min, and immediately place on ice.
The reactions can be stored at –20°C until PCR is started.

2. PCR

a. In a nuclease-free tube on ice, prepare a PCR master mix with all of the PCR reagents ***except the cDNA template***.
The reaction setup shown below is for a single 50 µL reaction, prepare enough PCR master mix for all of the samples in the experiment, and include 5% overage to compensate for pipetting error.

Mix well and centrifuge briefly.

Amount	Component
5 µL	10X PCR buffer
2.5 µL	dNTP Mix (2.5 mM each)
1.2 µL	gene specific primers (10 µM each)
1 Unit	thermostable DNA polymerase
*5 µL	cDNA template from RT reaction
to 50 µL	Nuclease-free Water

* 5–10 µL of the cDNA template can be added to the reaction.

b. On ice, dispense 45 µL of PCR master mix into nuclease-free reaction tubes, and add 5 µL of cDNA from the RT reaction. Mix well and centrifuge briefly.

PCR Cycling

See manufacturer's guidelines for information regarding cycling parameters for the thermostable DNA polymerase used.

	Stage	Reps	Temp	Time
Initial denaturation	1	1	95°C	5 min
Amplification	2	35–40	95°C	15 sec
			*annealing temp	30 sec
			68–72°C	1 min/kb
Final extension	3	1	72°C	5–10 min

* Use the annealing temp suggested by the primer design software, 60°C is often a good starting point for cycling temperature optimization.

Analyze the amplified product via agarose gel electrophoresis.

B. References

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C. Safety Information

The MSDS for any chemical supplied by Applied Biosystems or Ambion is available to you free 24 hours a day.

IMPORTANT

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

To obtain Material Safety Data Sheets

- Material Safety Data Sheets (MSDSs) can be printed or downloaded from product-specific links on our website at the following address: www.ambion.com/techlib/msds
- Alternatively, e-mail your request to: MSDS_Inquiry_CCRM@appliedbiosystems.com. Specify the catalog or part number(s) of the product(s), and we will e-mail the associated MSDSs unless you specify a preference for fax delivery.
- For customers without access to the internet or fax, our technical service department can fulfill MSDS requests placed by telephone or postal mail. (Requests for postal delivery require 1–2 weeks for processing.)

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.

D. Quality Control

Functional Testing

Following this protocol, a titration of the HeLa S3 Control RNA is used in qRT-PCR with the hGAPDH Control Primers and TaqMan detection. The standard curve created has an $r^2 \geq 0.98$.

Nuclease testing

Relevant kit components are tested in the following nuclease assays:

RNase activity
Meets or exceeds specification when a sample is incubated with 25 ng labeled RNA and analyzed by PAGE.

Nonspecific endonuclease activity
Meets or exceeds specification when a sample is incubated with 300 ng supercoiled plasmid DNA and analyzed by agarose gel electrophoresis.

Exonuclease activity
Meets or exceeds specification when a sample is incubated with 5 pmol labeled double-stranded DNA, followed by PAGE analysis.