RETROscript® Kit

Reverse Transcription for RT-PCR

Part Number AM1710

RETROscript® Kit

(Part Number AM1710)

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I. Introduction

A. Background

Reverse transcription and RT-PCR

The Polymerase Chain Reaction (PCR) was initially used to amplify target DNA sequences from complex mixtures such as genomic DNA. It is still used for that purpose, but it is also used extensively for amplification of RNA targets, typically mRNA or viral RNA. PCR requires a DNA template, so reverse transcriptase is used to copy the RNA into its complementary DNA sequence (cDNA). The cDNA then serves as template for amplification by PCR. The process of subjecting RNA to reverse transcription followed by PCR is commonly known as RT-PCR. RT-PCR can be used to generate inserts for cloning into plasmid vectors, to make templates for in vitro transcription, or to assess the mutational status of expressed sequences. RT-PCR can also be used to quantify the relative and absolute levels of mRNA targets, by using relative and/or competitive RT-PCR strategies.

The RETROscript® Kit

The RETROscript® Kit was designed to be convenient and complete, while using the highest quality components. There is also some flexibility in the kit design to accommodate many of the different experiments that use reverse transcription.

For convenience, the 4 deoxynucleotide triphosphates (dNTPs) are provided premixed (2.5 mM each). The kit also includes Positive Control PCR primers to amplify a conserved, constitutively expressed mRNA (S15, a ribosomal subunit protein), and a positive control RNA sample (from mouse liver) for use in troubleshooting. To load RT-PCR products on native agarose gels, a glycerol-based Gel Loading Solution is also included. It is optimized for high resolution of PCR products.

There are several steps in reverse transcription that can be done in different ways, and the RETROscript Kit leaves some of these decisions to the user. Both oligo$(dT)_{18}$ and random decamers are provided for use as first-strand primers. The RETROscript Kit also includes two alternative buffers for reverse transcription; the 10X RT Buffer may be used to maximize the total cDNA yield, or the 10X PCR Buffer can be used for both the reverse transcription and the PCR. The yield of specific RT-PCR products is usually similar using either buffer. Finally, procedures are provided for doing the RT-PCR reaction as either a 1- or 2-step procedure.

B. Reagents Provided with the Kit and Storage Conditions

The RETROscript Kit provides reagents for 40 reverse transcription reactions (20 µL each) and 40 PCRs (50 µL each). The thermostable DNA polymerase is not included.

Amount	Component	Storage
50 µL	Control Template RNA (mouse liver RNA 0.5 mg/mL)	below –70°C
200 µL	10X RT Buffer • 500 mM Tris-HCl, pH 8.3 • 750 mM KCl • 30 mM $MgCl_2$ • 50 mM DTT	–20°C
400 µL	10X PCR Buffer • 100 mM Tris-HCl, pH 8.3 • 500 mM KCl • 15 mM $MgCl_2$	–20°C
300 µL	dNTP mix (2.5 mM each dNTP)	–20°C
80 µL	Random Decamers (50 µM)	–20°C
80 µL	Oligo(dT) Primers (50 µM)	–20°C
40 µL	MMLV-RT (100 units/µL)	–20°C
40 µL	RNase Inhibitor (10 units/µL)	–20°C
50 µL	Control PCR Primers forward and reverse primers at 5 µM each	–20°C
1.0 mL	High Resolution Gel Loading Solution (5X)	–20°C
3.5 mL	Nuclease-free Water	any temp*

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

It is a good idea to briefly centrifuge the component tubes to collect the contents at the bottom before opening them.

C. Required Materials Not Included in the Kit

- RNA template
- Nuclease-free microcentrifuge tubes and pipet tips
- Thermostable DNA polymerase (recommended: SuperTaq™, recombinant thermostable DNA polymerase, Ambion P/N AM2050, AM2052)
- Material and equipment for analysis of RT-PCR products

D. Related Products Available from Applied Biosystems

*SuperTaq™ Polymerase P/N AM2050, AM2052	Thermostable DNA Polymerase (includes 10X buffers and dNTPs)
†SuperTaq™ Plus P/N AM2054, (50U) P/N AM2056, (250U)	Extended Range Thermostable DNA Polymerase Super Taq Plus has a proof reading activity, and produces significantly higher yields of PCR products than ordinary Taq polymerase (includes 10X buffers and dNTPs)
TURBO DNA-*free*™ Kit P/N AM1907	The TURBO DNA-*free* Kit employs the Ambion 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.
DNA-*free*™ Reagents P/N AM1906	DNase treatment and removal reagents. This product contains the Ambion 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.
Electrophoresis Reagents SeeweborprintcatalogforP/Ns	Ambion offers gel loading solutions, agaroses, acrylamide solutions, powdered gel buffer mixes, nuclease-free water, and RNA and DNA molecular weight markers for electrophoresis. Please see our catalog or our website (www.ambion.com) for a complete listing as this product line is always growing.

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II. RETROscript Procedure

A. Experimental Design

In the reverse transcription reaction, an oligonucleotide primer is annealed to an RNA population. Reverse transcriptase then extends annealed primers, creating a DNA copy (cDNA) that is the complement of the RNA sequence(s). There are several decisions to make before starting the procedure, these include choosing what kind of RNA to use and how to prepare it for reverse transcription, deciding what type of oligonucleotide primer to use, and selecting which variation of the procedure to follow. The next few sections discuss these choices, and supply some background information to help you make these decisions.

Total or poly(A) RNA?

The template RNA for reverse transcription may consist of total cellular RNA, mRNA purified by poly(A) selection, or RNA from some other source, for example viral RNA or ribosomal RNA.

RT-PCR

For RT-PCR, (i.e., making cDNA from specific mRNA targets and then amplifying the cDNA by PCR), it is usually not necessary to start with poly(A) RNA, even for rare messages. This is because, with a good set of primers, PCR is extremely sensitive.

cDNA libraries

For construction of cDNA libraries, it is common practice to use poly(A) RNA as template in the reverse transcription step. This will maximize the chance of getting cDNA representation of rare targets (i.e. very low-abundance mRNAs) in the library (see Farrell, 1993, Chapter 6). For recovery of cDNA clones of abundant mRNA species from cDNA libraries, it may be possible to use total RNA as template in the reverse transcription.

RNA purity and quality

Genomic DNA contamination and minus-RT controls

Because most RNA preparations contain some contaminating genomic DNA, it is important to include a minus-reverse transcriptase control in RT-PCR experiments. This will demonstrate that the template for the PCR product was cDNA, and not genomic DNA. Typically, the control is a mock reverse transcription, containing all the RT reagents, except the reverse transcriptase. Alternatively, to conserve kit reagents, the RNA can simply be added directly to the PCR as template. Since RNA cannot support amplification by PCR, any product from this reaction must come from contaminating DNA.

It is always a good idea to treat RNA preparations that will be used in RT-PCR with DNase I to remove genomic DNA. DNase I requires a buffer with divalent cations for optimal activity, yet if RNA is heated in

a solution containing divalent cations, it will be degraded. This is clearly a problem for reverse transcription, because the first step in the procedure is to heat denature the RNA to remove secondary structure. It is also important to remove the DNase enzyme which would degrade the primers and the cDNA if it were left in the reaction.

Contaminants

The most common methods for RNA purification use guanidinium thiocyanate and/or organic solvents such as phenol and chloroform. These compounds are protein denaturants, so they will inhibit Reverse Transcriptase if they are carried over to the RT reaction with the RNA. Some RNA isolation procedures also include a protease digestion step (typically with Proteinase K), and it is important to thoroughly remove the protease, to prevent it from digesting the Reverse Transcriptase. This is usually done by phenol/chloroform extraction, followed by alcohol precipitation.

RNA quality

The importance (or lack of importance) of using full-length RNA for reverse transcription depends on the application. For RT-PCR, some degradation of the RNA may be tolerated depending on the location of the target in the mRNA, and on what type of first-strand primers are used. Template mRNA that is completely free of any degradation is necessary for recovery of full length cDNA clones or amplification products. High-quality intact mRNA is also generally preferred for cDNA labeling applications. For amplification of relatively short internal regions of mRNA via RT-PCR, some degradation of the template RNA can be tolerated. When the mRNA template is partially degraded, it is better to use random decamers as first-strand primers, instead of oligo(dT), so that reverse transcription of the desired target region is not dependent on the presence of an intact 3' end (i.e., a poly(A) tail).

Procedure options

One step or two step RT-PCR

Typically RT-PCR is done with two separate reactions, first the reverse transcription, and then the PCR; this method will be referred to as the two step procedure. Alternatively, all of the components for RT and PCR can be added to a single tube at the same time. The mixture is first incubated at ~42°C for cDNA synthesis, and then the PCR temperature cycle is started to amplify the cDNA. This is commonly known as a one step RT-PCR.

The two step RT-PCR procedure may be more sensitive than the one step procedure for amplifying cDNA targets. Generally, it is also more efficient to use a two step rather than a one step RT-PCR strategy, because a single RT reaction will provide template for multiple PCRs. Finally, reagents are conserved with a two step RT-PCR, because the PCR is done in 50 µL, whereas the one step procedure requires a 100 µL

PCR volume to dilute out the RT components for more efficient amplification. We recommend using the two step RT-PCR procedure routinely. If the RT-PCR works consistently well with the two step procedure, then it may also work with the one step RT-PCR.

Choice of buffer for the reverse transcription

Two RT reaction buffers are supplied with the RETROscript Kit: 10X RT Buffer, and 10X PCR Buffer. Typically RT reactions using either of these buffers yield equal amounts of RT-PCR product. If you use the one step RT-PCR procedure (described in section IV.A on page 21), however, it is important to use the 10X PCR Buffer for the reverse transcription.

In traditional, two step RT-PCRs, the 10X RT Buffer often gives a higher yield of cDNA than 10X PCR Buffer. Typically, this difference is not reflected in the amount of specific product after the PCR.

Heat denaturation of the RNA template

Ordinarily, the template RNA is heated just before reverse transcription to denature (melt out) any secondary structure which could impede the reverse transcriptase from copying the RNA. To do this, the template RNA is typically mixed with the first-strand primers and the water and heated for a few min. It is important to keep the rest of the reaction components out of the heating step for two reasons. First, the heating step could denature and reduce the activity of the Reverse Transcriptase and the RNase Inhibitor. Also, the reaction buffers contain divalent cations which can cause RNA degradation when heated. The volume of components heated should be at least 5 µL, otherwise the entire contents of the tube may evaporate during the heating step.

Although the standard RT procedure calls for preheating the template RNA, for RT-PCR it is often possible to omit this step. Good yields of RT-PCR products can often be obtained, even for G+C-rich targets, without preliminary heating of the RNA template. In this case, the RT reaction is assembled at room temperature, but the incubation temperature during the reverse transcription reaction may be increased from 42°C to 44°C.

Primer Choice

Primers for reverse transcription

Generally reverse transcription reactions are primed with one of the following types of primers: random sequence oligonucleotides, oligo(dT), or an oligonucleotide that can hybridize with the specific RNA under study. The type of first-strand primer used for reverse transcription is mostly based on user preference. For cDNA library construction or cDNA labeling applications, oligo(dT) is almost always used to prime cDNA synthesis, so that the cDNA will start at the poly(A) tail. The greatest yield of RT product is usually obtained by using short random oligonucleotides to prime the reverse transcription,

but this yield advantage may not be seen after the PCR step. The other option for first-strand primers in RT-PCR experiments is to use a gene-specific primer, for example, the reverse (antisense) PCR primer. When template is limiting, final RT-PCR yield may be somewhat higher when the reverse transcription is primed with random primers as opposed to oligo(dT) or gene-specific primers (see Innis et al., Chapter 3, for further discussion).

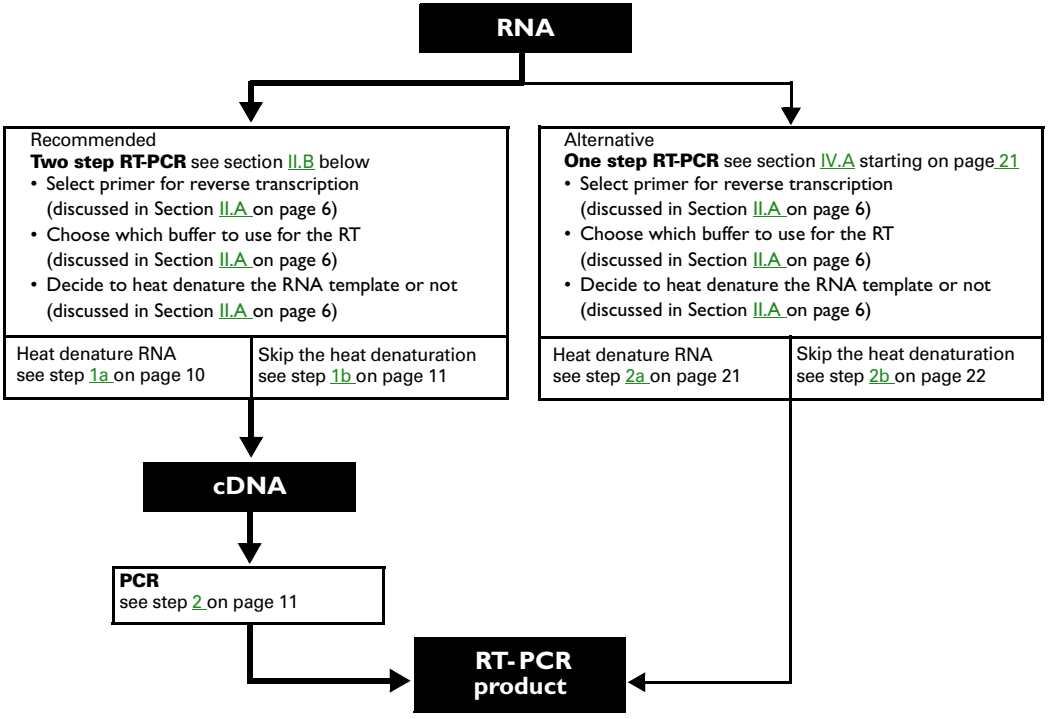
The concentration of both the Random Decamers and the Oligo(dT) Primers supplied with the RETROscript kit is 50 µM, yielding a final concentration of 5 µM in the RT reaction. If a gene-specific primer is used to prime the RT, its final concentration should be 0.25–5 µM (Innis et al, Chapter 3). For some targets, past experience may show that one type of primer is preferable, either for maximizing yield or for maximizing specificity of the RT-PCR product. For initial experiments, it may be useful to compare the yield and purity of RT-PCR products between reactions primed with random-sequence oligonucleotides, oligo(dT), and the reverse PCR primer.

PCR primers

For the most efficient design of PCR primers for RT-PCR applications, the use of primer design software is recommended. These programs are useful for avoiding primers with a tendency to generate unwanted side products, and they provide useful information about optimal annealing temperature and other important parameters to consider in optimizing the thermocycle profile. Another recommendation is to choose PCR primers that span at least one intron-exon boundary in the target mRNA, to prevent amplification of the target from contaminating genomic DNA that may be in the template RNA preparation, or to at least yield a product that would be distinguishable, by its larger size, from the intended RT-PCR product.

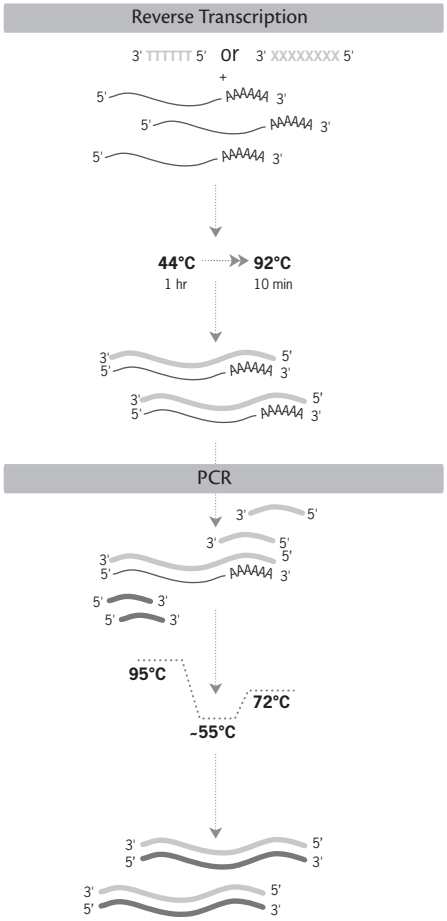
Finding the Instructions

With all the options in procedure, the following flow chart may be helpful; it shows some of the procedure options and where to find their instructions:



B. Two Step RT-PCR Procedure

Figure 1. Overview of Two Step RT-PCR



1. Reverse transcription reaction

When multiple reactions are done at the same time, prepare cocktails of ingredients to minimize pipetting. Also, you may want to include a negative control that lacks Reverse Transcriptase. The minus-RT control can be set up by either preparing an RT reaction with all the components except MMLV-RT, or to conserve reagents, by simply adding untreated RNA to the PCR in the next step of the procedure.

Two alternative procedures are presented below. Choose either *1a. RT with heat denaturation of the RNA* or *1b. RT without heat denaturation of the RNA* on page 11. A discussion of the two options is found in section II.A., on page 6.

1a. RT with heat denaturation of the RNA

Amount	Component
~1–2 µg	total RNA*
2 µL	Oligo(dT) or Random Decamers†
to 12 µL	Nuclease-free Water

* Use ~1–2 µg of total RNA, or 20–100 ng of poly(A) RNA. Less RNA can be used if the target is abundant. More RNA can also be used, but do not introduce >0.2 mM EDTA into the reaction along with the RNA.

† If you use a gene-specific first-strand primer, use 0.1–2 µL of a 50 µM stock to have a final concentration of 0.25–5 µM.

a. Mix, spin briefly, heat 3 min at 70–85°C.
The exact temperature is generally not critical, but 85°C might be more appropriate for targets that are GC-rich or that have a high degree of secondary structure.

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

c. Add the remaining RT components:

Amount	Component
2 µL	10X RT Buffer*
4 µL	dNTP mix
1 µL	RNase Inhibitor
1 µL	MMLV-RT†
20 µL	Final volume

* The 10X PCR Buffer may be used instead

† More MMLV-RT Reverse Transcriptase (2 µL) can be used for reactions with 2 µg of template RNA, or for synthesis of cDNA from very rare targets. Alternatively, for reactions with ≤20 ng template RNA, as little as one-tenth the amount of Reverse Transcriptase can be used. (Dilute in 1X reaction buffer just prior to use).

d. Mix gently, spin briefly. Incubate at 42–44°C for 1 hr. Elevating the incubation temperature to a maximum of ~55°C; may be beneficial for templates that don't reverse transcribe well at 42–44°C.

e. Incubate at 92°C for 10 min to inactivate the Reverse Transcriptase.

f. Store reaction at –20°C or proceed to the PCR (section II.B.2 on page 11).

1b. RT without heat denaturation of the RNA

Amount	Component
~1–2 µg	total RNA*
2 µL	Oligo(dT) or Random Decamers†
2 µL	10X RT Buffer ‡
4 µL	dNTP mix
1 µL	Placental RNase Inhibitor
1 µL	MMLV-RT **
to 20 µL	Nuclease-free Water

* Or use 20–100 ng of poly(A) RNA. Less RNA can be used if the target is abundant. More RNA can also be used, but do not introduce >0.2 mM EDTA into the reaction along with the RNA.

† If you use a gene-specific first-strand primer, use 0.1–2 µL of a 50 µM stock to have a final concentration of 0.25–5 µM.

‡ The 10X PCR Buffer may be used instead.

**More MMLV-RT Reverse Transcriptase (2 µL) can be used for reactions with 2 µg of template RNA, or for synthesis of cDNA from very rare targets.
Alternatively, for reactions with ≤20 ng template RNA, as little as 10% the amount of Reverse Transcriptase can be used. (Dilute in 1X reaction buffer just prior to use).

a. Mix gently, spin briefly. Incubate at 44°C for 1 hr. Elevating the incubation temperature to a maximum of ~55°C; may be beneficial for templates that don't reverse transcribe well at 42–44°C.

b. Incubate at 92°C for 10 min to inactivate the Reverse Transcriptase.

c. Store reaction at –20°C or proceed to the PCR.

2. PCR set-up

a. Negative Controls

There should be two negative controls among the PCRs.

- The minus-RT control from the previous step, or alternatively, untreated RNA can simply be subjected to PCR.
- A minus-template PCR, it should have all the PCR components, but use water as template instead of an aliquot of the cDNA (RT reaction). This control will verify that none of the PCR reagents are contaminated with DNA.

b. Assemble the following on ice, and mix gently:


NOTE

A hot start PCR may be beneficial in some cases, but it is not routinely necessary.

Amount	Component
1–5 µL	RT reaction (from step 1)
5 µL	10X PCR buffer
2.5 µL	dNTP mix
to 50 µL	Nuclease-free Water
2.5 µL	PCR primers (mixture with 5 µM of each primer)
1–2 U	Thermostable DNA Polymerase

3. Cycle as follows:

	Stage	Reps	Temp	Time
Initial denaturation	1	1	94–95°C	2–4 min
Amplification	2	30	94°C	20–30 sec
			annealing temp.*	20–30 sec
			72°C	40 sec–1 min†
Final extension:	3	1	72°C	5 min

* Start with the annealing temperature suggested by your primer design software. An annealing temperature of ~55°C used with the cycling times shown is often a reasonable starting point, but the optimal temperature and cycling times for your primer and template combination may need to be determined empirically.

† The rule of thumb is to use an extension time of 1 min per kilobase of target.

NOTE

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

C. Analysis of the Result

1. Agarose gel analysis of RT-PCR products

To analyze the RT-PCR reaction, run an aliquot (typically about 10 µL) on a native agarose gel in the presence of ethidium bromide, and visualize the product under UV light.

Gel type and staining

If you intend to use the High Resolution Gel Loading Solution supplied with the kit to load your samples, we recommend that you use TBE (Tris-borate-EDTA) as your gel solution and gel running buffer. For short RT-PCR products (<1 kb), a 2–2.5% agarose gel typically works well. Longer products may resolve better on a gel with slightly less agarose. We typically stain nucleic acids by adding 0.5 µg/mL ethidium bromide to the running buffer, or by adding ethidium bromide to the gel loading solution at a final concentration of ~10 µg/mL.

Gel loading solution

The High Resolution Gel Loading Solution (5X) supplied with the kit should be added to the sample to a final concentration of ~20%. It will increase band sharpness and resolution, especially for small fragments, compared to standard gel loading solutions. The sample may be diluted with water or buffer to increase the loading volume if desired.

2. Negative controls

Both the minus-RT and the minus-template negative controls should have no visible reaction products after the PCR. If any bands appear in these lanes, see section III.3 on page 19, for more information.

3. Storage and/or purification of the RT-PCR

The remaining RT-PCR can be stored at –20°C. If desired, the product can be further purified, for example by gel electrophoresis and electroelution, column filtration, organic extraction, or alcohol precipitation. For some purposes, such as digestion with common restriction enzymes, or use as transcription template, purification of the RT-PCR product is not required.

III. Troubleshooting

A. Using the Positive Control

The positive control reaction can be used to demonstrate that the kit components, and the user-supplied PCR reagents and equipment are functioning as expected. The RETROscript Kit includes an aliquot of Control Template RNA and a set of Positive Control PCR Primers.

1. Positive Control Primers

The Control PCR Primers amplify a 361 bp, highly conserved region of a constitutively expressed "housekeeping" gene, rig/S15, which encodes a small ribosomal subunit protein (Inoue et al, 1987; Kitagawa et al., 1991). The primers correspond to the human S15 sequence, and they should work well with human, mouse, hamster, and rat mRNA, and probably with many other vertebrate S15 sequences as well. Because it is a constitutively expressed message, the S15 RNA will be present in RNA isolated from any tissue.

Table 1. Positive Control Primer sequences

forward primer	5'-TTCCGCAAGTTCACCTACC
reverse primer	5'-CGGGCCGGCCATGCTTTACG

2. Uses of the Control PCR Primers

Check your reagents and equipment

The Control PCR Primers can be used with the Control Template RNA in the kit to make sure that the thermostable DNA polymerase, and/or thermal cycler are working as expected. To model the situation for RT-PCR of a rarer transcript, the amount of Control Template RNA used in the reverse transcription step can be reduced from 1 µg to 0.05 µg.

Check your RNA

The Control PCR Primers can be used to verify that an RNA preparation can support RT-PCR (if the RNA contains an S15 mRNA with the Control PCR Primer binding sites).

Check for DNA contamination in RNA

The Control PCR Primers span two introns; the forward primer binds in the second exon, and the reverse primer binds in the fourth exon. Despite this, they amplify a product of the same size from genomic DNA (mouse or human) and from cDNA. This is presumably due to the presence of processed pseudogenes in these genomes. This makes the Control PCR Primers useful for detecting DNA contamination in RNA preps used for RT-PCR. A 2–5 µL aliquot of mouse or human RNA can be added directly to a PCR (without prior reverse transcription); if a product is amplified with the Control PCR Primers, then genomic DNA is probably present in the RNA.

3. Positive control procedure

a. Reverse transcription

Do the positive Control RT-PCR as described in step *1a. RT with heat denaturation of the RNA* on page 10. Use 1–2 µL of the Control Template RNA in the reverse transcription [or use only 2.5 µL of a 1:100 dilution of the Control Template RNA (50 ng) if you want to mimic the RT-PCR of a rare target].

b. PCR setup

Follow the instructions in section *II.B.2. PCR set-up* on page 11. ***Use 5 µL of the RT in the PCR.*** It is a good idea to include the positive and negative controls described in that section.

c. PCR cycle

	Stage	Reps	Temp	Time
Initial denaturation	1	1	95°C	1 min
Amplification	2	30	94°C	30 sec
			55–59°C	30 sec
			72°C	30 sec
Final extension:	3	1	72°C	5 min

NOTE

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

d. Visualization of the results

Mix 10 µL of the PCRs with 2 µL of the High Resolution Gel Loading Solution, and electrophorese on a 2–2.5% agarose/TBE gel stained with ethidium bromide (see section II.C.1 on page 12 for more details).

4. Expected result of the positive control RT-PCR

The positive control RT-PCR should yield a single 361 bp product that is easily visible. If the expected result is not seen, there may be a problem with the PCR reagents, the thermal cycler, or with one of the RETROscript Kit components.

B. Low Yield of RT-PCR Product

Low yield of desired products in RT-PCR may result from degraded or impure sample RNA which can limit the efficiency of the RT reaction and reduce yield. Problems in the PCR step can also result in low product yield, co-amplification of non-specific products, or poor reproducibility of results. In this section, possible causes of low to no yield of RT-PCR products are discussed as well as suggestions for how to circumvent these problems.

1. Do the positive control

First, determine whether the kit is working properly by doing the positive control reaction (see section III.A on page 14). If the positive control does not show the expected 361 bp PCR product after RT-PCR, consider repeating the experiment to make sure that all the steps were completed properly. The positive control would fail if there were a problem with the PCR reagents, the thermal cycler, or with one of the RETROscript kit components.

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 alcohol with a drawn-out glass pipette, a fine-bore needle, or a fine-bore pipet tip.

Mixing experiment to test for inhibitors of reverse transcription

You can test for inhibitors of reverse transcription in your RNA by doing a mixing experiment. To do this, set up RT reactions with the Control Template RNA and your RNA both mixed and alone. Add a trace amount of radiolabeled nucleotide to the reactions, and analyze the results on a denaturing polyacrylamide gel (8 M urea, 5% acryl).

The expected result (if the RNA is intact) is that radiolabeled nucleotide will be incorporated into high molecular weight material, heterogeneous in size, with the majority running larger than ~800 bases. If the size or amount of the cDNA produced from the mixture of the Control Template RNA and your RNA is reduced compared to the reaction with only control template RNA, this suggests that there may be an inhibitor present in the experimental RNA preparation. Do an organic extraction and an alcohol precipitation as described above to clean up your sample. If adding your RNA to the Control Template RNA has no effect on the product of reverse transcription from the Control RNA, then the problem is with your RNA (see below).

3. Check the quality of your sample RNA

a. Denaturing agarose gel electrophoresis

The overall quality of a total RNA preparation can be assessed by electrophoresis on a denaturing agarose gel. Protocols for analysis of total RNA on denaturing agarose gels are included in most basic molecular biology manuals. To facilitate visualization of the RNA under UV light, add 5 µg/mL ethidium bromide to the running buffer, or add ethidium bromide to the gel loading solution at a final concentration of ~10 µg/mL.

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. The ribosomal RNA bands should be fairly sharp and intense. The upper band should be about twice as intense as the lower band. Smaller, more diffuse low molecular weight RNAs (tRNA and 5S ribosomal RNA) may also be present depending on how the RNA was isolated. A diffuse smear of mRNA should be seen migrating between the 18S and 28S ribosomal bands. DNA contamination, if it is present, will form a high molecular weight smear or band migrating above the larger ribosomal RNA band. Degradation of the RNA will be reflected by smearing of ribosomal RNA bands.

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

b. Test the RNA in RT-PCR with the Positive Control PCR Primers

The control primers supplied with the kit can be used with RNA from many vertebrate species. The primers are designed to bind to a constitutively expressed target – the message for the S15 small ribosomal subunit protein. Section III.A on page 14 has instructions for setting up the control reaction.

If the expected RT-PCR product is seen with the control primers using ~1 µg of user-supplied RNA, but is not seen when the amount of RNA in the RT is decreased to ~50 ng, the template may be partially degraded such that rare mRNA targets cannot be amplified.

4. Use more template RNA in the reverse transcription

Use up to 5 µg of total RNA per 20 µL RT reaction. If the RT-PCR yield is still insufficient, it may help to use poly(A) RNA, rather than total RNA, as template for reverse transcription. The optimal amount of poly(A)-selected RNA to use should be determined empirically. Keep in mind that the copy number of an average transcript in 5 µg total RNA is roughly similar to that present in 160 ng of poly(A)-selected RNA.

5. Use a different primer for the RT

It sometimes help to use another primer type for the reverse transcription. If Oligo(dT) Primers were used in an unsuccessful RT-PCR, try using Random Decamers in subsequent attempts.

6. Increase incubation temperature of reverse transcription step

A potential reason that a particular RT-PCR product is not obtained is that secondary structure in the mRNA template prevented it from being reverse transcribed into cDNA. In this case, it may help to do the reverse transcription reaction at up to 55°C.

7. The PCR requires optimization

a. Primer annealing temperature is not optimal
Try several annealing temperatures to identify the one that works the best.

b. Try a nested 2-Step amplification strategy
Another option for increasing the yield and specificity of RT-PCR reactions is to use a nested, two-step strategy. Nested primers that bind to sites internal to the product of the first PCR are used in a second reaction, with an aliquot of the first-step PCR as template. See section IV.B on page 23 for details on this strategy.

c. Optimize the Mg^{++} concentration in the PCR
PCR yields can sometimes be improved by fine-tuning the magnesium concentration in the reaction. Try altering the magnesium level by increasing and decreasing it in ~0.2 mM increments. If the 10X RT Buffer was used in the reverse transcription step, and 5 µL of the RT reaction is used in a 50 µL PCR containing 1.5 mM $MgCl_2$ from the 10X PCR Buffer, the final magnesium concentration in the reaction will be 1.8 mM, which may be too high for amplifying some targets.

C. Spurious RT-PCR Products

1. Non-specific priming

Sometimes unexpected products in an RT-PCR come from non-specific priming of unrelated cDNA sequences during the PCR. Raising the stringency of the PCR by increasing the annealing temperature can often improve results. If neither the minus-RT nor the minus-template control reactions yields PCR product, then the RT-PCR is producing reverse transcriptase- and template-dependent spurious products (e.g. spurious products from RNA). In this case, check the performance of the thermocycler, and make sure that the PCR reactions are set up on ice.

2. PCR products from DNA contamination

Spurious amplification products are often from contaminating genomic DNA. If PCR primers are designed to flank at least 1 intron, then PCR products from contaminating genomic DNA will typically be a different size than products from cDNA. Occasionally a processed (introns spliced out) pseudogene may be present in genomic DNA. If this is the case (as it is for S15, the positive control, in human and mouse), even primers that flank an intron will produce the same PCR product from both genomic DNA and cDNA. One way to prove that products of the RT-PCR are due to DNA contamination is to include both minus-RT and minus-template control reactions routinely.

a. Minus-RT control

Omit the reverse transcriptase from the RT reaction, but otherwise do the RT-PCR reaction as usual. Alternatively, simply skip the RT step completely, and add RNA to the PCR. Since RNA cannot typically serve as a template for thermostable DNA polymerase, any product amplified from RNA is probably from a DNA template. Be aware, however, that thermostable DNA polymerases, including native Taq, have been known to have 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.

If the minus-RT control makes a PCR product, the recommended course of action is to treat the RNA preparation with DNase, and then to inactivate the enzyme. See *III.C.3. Products in the minus-RT control* on page 19.

If bands are not seen in the minus-RT control, the spurious bands in RT-PCR may represent alternatively spliced forms of the transcript or amplification of a fragment from a related message. Cloning and sequencing of the PCR products would resolve this question. Alternatively, design primers to a unique region of the transcript (e.g. the 3' untranslated region), and try these in the PCR.

b. Minus-template control

Do a PCR with all ingredients except template. If product is produced, one or more reagents are contaminated with DNA (typically from a previous reaction). See section *III.C.4. Products in the minus-template control* on page 20.

3. Products in the minus-RT control

If PCR products are produced by the minus-RT control, it is probably because there was DNA contamination in the RNA preparation. There is a report, however, 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.

To eliminate contaminating DNA, treat the template RNA with DNase. The Ambion DNA-*free*™ Kit (P/N AM1906) is ideally suited for this purpose, because in addition to high-quality DNase, a novel reagent is provided to rapidly inactivate the DNase, and to remove divalent cations (carried over from the DNase reaction buffer) from the RNA.

Note that RNA should not be heated (for example to inactivate DNase) in the presence of divalent cations, because it can cause significant degradation. Also, to minimize the effects of DNA contamination in RT-PCR experiments, PCR primers should be designed to hybridize in separate exons, so that products amplified from DNA can be distin-

guished by their size from the intended RT-PCR products. For more information on eliminating DNA contamination, please request Ambion Technical Bulletin #176.

4. Products in the minus-template control

Contamination of PCR reagents, pipettors, and benchtops with DNA is quite common. Unfortunately the only way to remedy contaminated reagent(s) is to replace them.

Careful laboratory practices are essential to avoid contaminating reactions with PCR products. Keep concentrated DNA solutions (PCR products, plasmid prep, etc.) away from the area where PCRs are assembled. Clean the lab bench and the pipettors routinely with Ambion DNA*Zap*™ Solution (P/N AM9890) or another DNA de-contamination product. Use barrier tips to pipette PCR reagents, and store completed PCR reactions in a different location than the PCR reagents.

It is always a good idea to routinely include a minus-template negative control reaction with experimental PCRs. If minus-template controls routinely yield PCR products, more stringent steps may be taken to control contamination (see Yap, et al. 1994).

IV. Additional Procedures

A. One step RT-PCR

One step RT-PCR generally works well for amplifying targets that are reasonably abundant; yields of rare targets may be reduced, compared to using the two step procedure.

When multiple reactions are carried out, prepare cocktails of appropriate ingredients to minimize pipetting.

As in the two step RT-PCR procedure, it is often possible to omit the preheating of total RNA step prior to assembling the reaction. Use one of the two alternative procedures presented below. Choose either *2a. One step RT-PCR setup with heat denaturation of the RNA* or *2b. One step RT-PCR setup without heat denaturation of the RNA* on page 22. A discussion of the 2 options is found in section II.A. on page 6.

1. Negative Controls

There should be 2 negative controls included in the reactions. One is a minus-RT control where an aliquot of an RT reaction without Reverse Transcriptase is used as template, or where RNA is added directly to the PCR. The second is a minus-template PCR, containing all of the reaction components, but using water as template (instead of RNA). The minus-template control will verify that none of the RT or PCR reagents are contaminated with DNA.

2a. One step RT-PCR setup with heat denaturation of the RNA

Amount	Component
1–2 µg	Template RNA*
6.5 µL	dNTP mix
2 µL	First-strand primers†
73 µL	Nuclease-free Water

* Use this amount of total RNA, or 20–100 ng of poly(A) RNA. Less RNA can be used if the target is abundant. More RNA can also be used, but do not introduce >0.2 mM EDTA into the reaction along with the RNA.

† If you use a gene-specific first-strand primer, use 0.1–2 µL of a 50 µM stock to have a final concentration of 0.25–5 µM.

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

b. Remove tube(s) to ice; spin briefly; replace on ice.

c. Add the remaining RT-PCR components, then mix gently and spin briefly.

Amount	Component
10 µL	10X PCR Buffer
1 µL	RNase Inhibitor
5 µL	PCR primers (mixture with 5 µM of each primer)
1–2 µL	Reverse Transcriptase
1–2 U	Thermostable DNA polymerase

2b. One step RT-PCR setup without heat denaturation of the RNA

Assemble the following, and mix gently:

Amount	Component
1–2 µg	Template RNA*
6.5 µL	dNTP mix
2 µL	First-strand primers†
73 µL	Nuclease-free Water
10 µL	10X PCR Buffer
1 µL	RNase Inhibitor
5 µL	PCR primers (mixture with 5 µM of each primer)
1–2 µL	Reverse Transcriptase
1–2 U	Thermostable DNA polymerase

* Use this amount of total RNA, or 20–100 ng of poly(A) RNA. Less RNA can be used if the target is abundant. More RNA can also be used, but do not introduce >0.2 mM EDTA into the reaction along with the RNA.

† If you use a gene-specific first-strand primer, use 0.1–2 µL of a 50 µM stock to have a final concentration of 0.25–5 µM.

3. Incubate as follows:

	Stage	Reps	Temp	Time
Reverse transcription	1	1	42–44°C	1 hr
RT inactivation and initial denaturation	2	1	94°C	5 min
Amplification	3	30	94°C	20–30 sec
			annealing temp.*	20–30 sec
			72°C	40 sec–1 min†
Final extension	4	1	72°C	5 min

* Start with the annealing temperature suggested by your primer design software. An annealing temperature of ~55°C used with the cycling times shown may be a reasonable starting point, but the optimal temperature and cycling times for your PCR may need to be determined empirically.

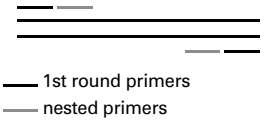
† The rule of thumb is to extend for 1 min per kb of target in the PCR cycle.

For amplification of extremely rare mRNA targets, it may be beneficial to use mRNA instead of total RNA. Ambion Poly(A)Purist™ Kits offer the highest mRNA recovery of any commercially available kit.

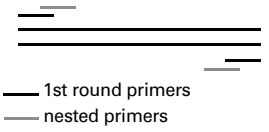
RNA should be stored in small aliquots at –20°C, in a non-frost-free freezer, or at –80°C for long-term storage. For amplification of relatively short sub-regions of mRNA targets, total RNA preparations that are partially degraded can often be used successfully.

B. Nested PCR to Amplify Rare Targets

Nested PCR



Partially nested PCR



Hemi-nested PCR

Low yields of RT-PCR products can often be improved by doing a second round of PCR, using primers that are "nested" within the amplified first-round product. This strategy has been particularly useful for amplifying cDNA targets from lymphocyte RNA, when the expression of the gene is generally thought to be limited to other tissues not readily accessible for RNA isolation. This phenomenon has been termed "ectopic expression" (Sarkar and Sommer 1989) or "illegitimate transcription" (Chelly et al. 1989), and has been exploited for the mutational analysis of many different genes, including CTFR (Fonknechten et al. 1992), cardiac myosin (Watkins et al. 1992), Factor VIII (Bidichandani et al., 1995), alpha1 collagen (Chan and Cole 1991) and neurofibromatosis (Martinez et al. 1995). If it is not convenient to use nested primers which are completely internal to the first-round primer binding sites, they can be partially nested, i.e. their sequence(s) may partially overlap that of the original primers, but the 3' ends of the second-round primers should extend past the 3' ends of the original primers. Hemi-nested strategies can also be used, where only one primer used for the second PCR is nested. Even if the product of the first-round amplification cannot be detected by ethidium bromide staining, there may be enough material to serve as template for the nested reaction.

For the nested PCR, use an aliquot of the first-round product (~1–10 µL) in a 50 µL PCR, with the nested primers. The thermocycle profile may need to be re-optimized for the nested primers. Besides improving the yield from PCR, an additional benefit of nested PCR is that the specificity of the reaction may be increased, since any unwanted products amplified in the first round will not generally contain the primer binding sites for the nested primers. One situation where we have seen a definite benefit of using a nested RT-PCR strategy is in amplifying the p53 coding region from tumor samples. Using a panel of 12 total RNA samples isolated from breast and lung tumors, the yield of specific ~800 bp first-round RT-PCR product (containing p53 exons 4–10) was highly variable between the samples, ranging from invisible to easily detectable. However, when 1–5 µL of the first-round RT-PCR product was used as template in a second round of PCR, using nested primers, all samples yielded easily detectable, fairly uniform amounts of the expected product.

V. Appendix

A. References

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B. 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.

C. Quality Control

Functional testing

All components are tested in a functional RETROscript reaction using 20 ng of mouse liver total RNA for amplification with the positive control primers. PCR products are assessed on a 2% agarose gel.

Nuclease testing

Relevant kit components are tested in the following nuclease assays:

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

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

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

Protease testing

Meets or exceeds specification when a sample is incubated with protease substrate and analyzed by fluorescence.