INTRODUCTION

This protocol uses a single thermostable RNA polymerase to perform high-specificity RT-PCR. A high-temperature RT reaction is followed by PCR amplification of the cDNA using a single thermostable polymerase, the GeneAmp AccuRT RNA PCR enzyme from Applied Biosystems. The high temperature of the RT reaction enhances the specificity of primer binding and also reduces secondary structure in the template, thereby increasing the efficiency of polymerization. For hot-start reactions, the GeneAmp AccuRT Hot-start RNA PCR enzyme, also from Applied Biosystems, is recommended. This enzyme is associated with an aptamer that binds and specifically inactivates the polymerase at temperatures below 55°C. The reaction conditions for conventional and hot-start RT-PCR are provided in Step 1.

Both GeneAmp enzymes are compatible with the dUTP/uracil N glycosylase (UNG) system for the prevention of carryover contamination. Reaction conditions the use of UNG technology are described in Step 2 of the procedure.

The success of this procedure relies on the use of high-quality RNA templates.

MATERIALS

Reagents

AmpErase uracil-N-glycosylase (UNG): 1 unit/µl (Applied Biosystems)

For use with UNG technology to prevent carryover contamination. See Step 2.

dNTP solutions, each at 10 mM (Applied Biosystems, containing 20 mM dUTP in place of dTTP)

Ethidium bromide

RNase-free H₂O

SYBR Green I Dye (Molecular Probes)

Required if performing dye-binding, real-time quantitative PCR.

GeneAmp AccuRT RNA PCR Kit or GeneAmp AccuRT Hot Start RNA PCR kit (Applied Biosystems, containing either the GeneAmp AccuRT RNA PCR Enzyme or GeneAmp AccuRT Hot Start RNA PCR, 5X AccuRT Buffer, and 25 mM magnesium acetate)

Oligonucleotide primers: 15 µM in 10 mM Tris-Cl, pH 8.3

RNA template
Generally this should be stored in carrier RNA such as 30 µg/ml Escherichia coli rRNA or poly(rA) in either RNase-free H2O or TE buffer, pH 7.0.

The preparation of high-quality RNA for study is of paramount importance. There are numerous commercial products on the market, as well as detailed protocols available (e.g., *Purification of RNA from Cells and Tissues by Acid Phenol-Guanadinium*).

**Equipment**

Electrophoresis apparatus for agarose gels (see *Agarose Gel Electrophoresis*)

GeneAmp PCR System 9700 or 2700 (Applied Biosystems), or equivalent

Real-Time quantitative PCR detection system (e.g., Applied Biosystems), optional; see Step 3.

GeneAmp 5700 Sequence Detection System or ABI PRISM 7000, 7700, or 7900HT

Sequence Detection Systems or the Roche Applied Science LightCycler

MicroAmp reaction tubes (Applied Biosystems), or equivalent

**METHOD**

**Combined RT and PCR Amplification**

1. For reactions performed with dTTP and without UNG:
   i. Combine the following reagents to make a master mix:

<table>
<thead>
<tr>
<th>µl</th>
<th>Final concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>RNase-free H2O</td>
<td>28.66</td>
</tr>
<tr>
<td>5X AccuRT Buffer</td>
<td>10.00</td>
</tr>
<tr>
<td>10 mM dATP</td>
<td>1.00</td>
</tr>
<tr>
<td>10 mM dCTP</td>
<td>1.00</td>
</tr>
<tr>
<td>10 mM dGTP</td>
<td>1.00</td>
</tr>
<tr>
<td><strong>10 mM dTTP</strong></td>
<td>1.00</td>
</tr>
<tr>
<td>AccuRT or AccuRT Hot Start Enzyme (3.75 units/µl)</td>
<td>2.00</td>
</tr>
<tr>
<td>15 µM &quot;upstream&quot; primer</td>
<td>0.67</td>
</tr>
<tr>
<td>15 µM &quot;downstream&quot; primer</td>
<td>0.67</td>
</tr>
<tr>
<td><strong>25 mM magnesium acetate</strong></td>
<td>3.00</td>
</tr>
</tbody>
</table>

   Reagents in bold are required in different concentrations for reactions performed with dUTP and UNG.

   ii. Gently vortex the master mix, add 1.00 µl of RNA (up to 1 µg; see Troubleshooting), and gently vortex the entire reaction mix.

   iii. Perform the RT reaction and PCR amplification consecutively in a GeneAmp PCR System 9700 or 2700 as follows:

   - 30 minutes at 65°C (RT step)
   - 1 minute at 95°C
15 seconds at 95°C and 30 seconds at 65°C for 40 cycles (PCR amplification)

7 minutes at 65°C

iv. Analyze the reactions directly (see Step 3) or store at –20°C.

2. For reactions performed with dUTP and UNG (for Carryover Contamination Control and UNG-mediated Hot Start)
   
i. Combine the following reagents to make a master mix:

<table>
<thead>
<tr>
<th>µl</th>
<th>Final concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>25.66</td>
<td>-</td>
</tr>
<tr>
<td>10.00</td>
<td>1X</td>
</tr>
<tr>
<td>1.00</td>
<td>200 µM</td>
</tr>
<tr>
<td>1.00</td>
<td>200 µM</td>
</tr>
<tr>
<td>1.00</td>
<td>200 µM</td>
</tr>
<tr>
<td>1.00</td>
<td>400 µM</td>
</tr>
<tr>
<td>2.00</td>
<td>7.5 units/50 µl</td>
</tr>
<tr>
<td>1.00</td>
<td>1.0 unit/50 µl</td>
</tr>
<tr>
<td>0.67</td>
<td>0.20 µM</td>
</tr>
<tr>
<td>0.67</td>
<td>0.20 µM</td>
</tr>
<tr>
<td>5.00</td>
<td>2.5 mM</td>
</tr>
</tbody>
</table>

Reagents in bold are required in different concentrations for reactions performed with dTTP and without UNG.

ii. Gently vortex the master mix, add 1.00 µl of RNA (up to 1 µg; see Troubleshooting), and gently vortex the entire reaction mix.

iii. Perform the RT reaction and PCR amplification consecutively in a GeneAmp PCR System 9700 or 2700 as follows:

   2 minutes at 50°C (UNG step)

   30 minutes at 65°C (RT step)

   1 minute at 95°C

   15 seconds at 95°C and 30 seconds at 65°C for 40 cycles (PCR amplification)

   7 minutes at 65°C

iv. Analyze the samples (see Step 3) immediately after PCR amplification, or store at –20°C.

   *Storage at –20°C will inactivate any remaining UNG. This is not a prerequisite to analysis, but it will destroy amplicons in reactions that are allowed to sit between room temperature and <55°C.*

**Analysis of Reaction Products**

3. Analyze the amplification products on a 2% agarose gel and stained with 0.5 µg/ml ethidium bromide, or subject them to real-time quantitative PCR using TaqMan technology with direct detection via ethidium
bromide or SYBR Green I Dye.

**TROUBLESHOOTING**

**Problem:** Generally poor results from amplification (including low yields and production of nonspecific products).

**[Step 3]**

**Solution:** Make sure that all reagents are stored correctly and are completely thawed before use. Pipette reagents slowly and always follow the recommended order of reaction assembly. It is especially important to add the magnesium acetate either just before adding template or immediately afterward to minimize generating nonspecific extension products when the GeneAmp AccuRT Hot Start RNA PCR Enzyme is not used. Avoid adding high concentrations of EDTA or other metal ion chelators with the primers or template.

Ensure that the template RNA is of the highest quality. If the starting concentration is low, the addition of carrier nucleic acid (e.g., *E. coli* rRNA or poly[rA]) will prevent nonspecific binding of RNA to sample tubes, etc. Low concentrations (copy number or molecules) of target RNA may require up to 35 or more cycles of PCR amplification to produce sufficient product for analysis. High levels of total RNA (>1 µg) may cause inhibition and should be avoided.

Make sure that reaction conditions are suitable for the primers chosen. Note that the annealing temperature cannot be significantly decreased when using the GeneAmp AccuRT Hot Start RNA PCR Enzyme because of the presence of the aptamer, which will decrease enzymatic activity at lower temperatures. Elevated temperatures also maintain the specificity of the reaction. The optimum temperature for the GeneAmp RNA PCR Enzymes is 65-80°C, depending on template and reaction buffer composition.

High-temperature reverse transcription using *rTth* pol and *T. Z05* DNA polymerase helps to alleviate many of the problems typically encountered when amplifying RNA targets. However, increased metal ion-catalyzed hydrolysis of template RNA is observed at the elevated temperatures (Brown 1974) used with these enzymes. Therefore, factors such as increased enzyme activity and reaction specificity, as well as the reduction of RNA secondary structure, must be balanced with target degradation.

Remember that the length of the target sequence will define the RT reaction time and the PCR amplification extension time. However, RT reaction times greater than 60 minutes are generally counterproductive. The thermal cycling parameters provided in the protocol were chosen for optimal performance with amplicons 200-500 nucleotides in length.

The reagent concentrations provided in the protocol were chosen for optimal performance with multiple targets and may need to be adjusted. If the dNTP concentrations are modified, make sure that they remain equimolar (the exception being dUTP) to minimize misincorporation of nucleotides. When using dUTP, a final concentration of 400 µM dUTP should be used, and the dATP, dCTP, and dGTP should remain at 200 µM in all reactions. The optimal magnesium acetate concentration should be determined empirically by titration between 1 and 4 mM, keeping in mind that the Mg ++ requirement may vary depending on the total dNTP concentration and on the primers and template used. In general, 1.5 mM magnesium acetate is a good starting place when using dTTP, and 2.5 mM magnesium acetate is the preferred starting point when using dUTP. Reaction conditions are extremely important in the overall fidelity achieved in an RT-PCR.

The addition of a final, total 1% DMSO concentration to the reaction often reduces nonspecific product formation and improves the results with the GeneAmp AccuRT RNA PCR Enzymes. However, the DMSO, glycerol, and/or other cosolvent concentrations present in the reaction must be taken into account when designing primers, because they effectively lower the *T*<sub>m</sub> of the oligonucleotides. The use of sequence-specific oligonucleotide primers is preferred, especially when reaction specificity is critical. Primers are typically 15-30 nucleotides in length and should be purified by gel electrophoresis or high-performance liquid chromatography/ ion-exchange chromatography. Primer sequences should not be complementary within themselves or to each other, particularly at the 3' terminus, to reduce primer artifact formation. Another common technique to reduce terminal overlap of primers and primer artifacts, especially in complex multiplex PCR amplifications, is to create primers that have the dinucleotide "AA" at their 3' termini.
Finally, it is critical to realize the enormous amplification of products produced during PCR. Low levels of DNA contamination, especially from previous PCR amplifications, samples with high DNA levels, or positive control templates can result in product formation, even in the absence of intentionally added template DNA. The use of the dUTP/UNG carryover prevention system is strongly recommended, especially for clinical diagnostic applications.

REFERENCES


**Caution**

Ethidium bromide

Ethidium bromide is a powerful mutagen and is toxic. Consult the local institutional safety officer for specific handling and disposal procedures. Avoid breathing the dust. Wear appropriate gloves when working with solutions that contain this dye.

**Caution**

SYBR Green

SYBR Green I is supplied by the manufacturer as a 10,000-fold concentrate in DMSO which transports chemicals across the skin and other tissues. Wear appropriate gloves and safety glasses and decontaminate according to Safety Office guidelines. See DMSO.

**Recipe**

Ethidium bromide
Add 1 g of ethidium bromide to 100 mL of H₂O. Stir on a magnetic stirrer for several hours to ensure that the dye has dissolved. Wrap the container in aluminum foil or transfer the 10 mg/mL solution to a dark bottle and store at room temperature.

---

**Recipe**

**TE buffer, 10X**

- 100 mM Tris-Cl (desired pH)
- 10 mM EDTA (pH 8.0)

Sterilize solutions by autoclaving for 20 min at 15 psi (1.05 kg/cm²) on liquid cycle. Store the buffer at room temperature.

---

**Recipe**

**Tris-Cl**

- Tris base
- HCl

To prepare a 1 M solution, dissolve 121.1 g of Tris base in 800 mL of H₂O. Adjust the pH to the desired value by adding concentrated HCl.

<table>
<thead>
<tr>
<th>pH</th>
<th>HCl</th>
</tr>
</thead>
<tbody>
<tr>
<td>7.4</td>
<td>70 mL</td>
</tr>
<tr>
<td>7.6</td>
<td>60 mL</td>
</tr>
<tr>
<td>8.0</td>
<td>42 mL</td>
</tr>
</tbody>
</table>

Allow the solution to cool to room temperature before making final adjustments to the pH. Adjust the volume of the solution to 1 L with H₂O. Dispense into aliquots and sterilize by autoclaving.

If the 1 M solution has a yellow color, discard it and obtain Tris of better quality. The pH of Tris solutions is temperature-dependent and decreases ~0.03 pH units for each 1°C increase in temperature. For example, a 0.05 M solution has pH values of 9.5, 8.9, and 8.6 at 5°C, 25°C, and 37°C, respectively.