**Notes**

1. In this example, the final volume of PCR mix should be sufficient for 100µl reactions once the cDNA volume is added. The volume of each component may be scaled for reactions of less than 100µl. Scale up volumes to accommodate the total number of PCR amplifications being performed.
2. The amount of reverse transcription reaction used in the PCR may be modified after experimental optimization.
3. The amounts of magnesium and dNTPs and volume of reaction buffer added to the PCR vary, depending on how much RT reaction is used as template. For example, for a 100µl PCR that contains 20µl of RT product, 8µl of 10X thermophilic polymerase reaction buffer is added to support the 80µl PCR mix addition. If 5µl of RT reaction were added to 95µl of PCR mix, 9.5µl of 10X thermophilic polymerase reaction buffer would be needed. Similar considerations must be given to the magnesium and dNTP additions. This example details the amplification conditions recommended to amplify either 1µl or 20µl of the cDNA synthesized in the positive control reverse transcriptase reactions containing the 1.2kb Kanamycin Positive Control RNA template in a 100µl PCR.

## VIII. Troubleshooting PCR and RT-PCR

| Symptoms | Solutions |
|---|---|
| Low yield or no amplification product (PCR or RT-PCR) | Template was degraded. Verify template integrity by electrophoresis. Repurify the DNA or RNA template if the nucleic acid appears degraded. |
| | Too much or too little template was used. Verify template concentration by comparing the staining intensity of the DNA template after agarose gel electrophoresis and ethidium bromide staining with that of DNA standards with known concentrations. |
| | Inhibitor was present in sample. Reduce template volume in the reaction. Perform an ethanol precipitation to remove inhibitors. Some common inhibitors are listed in the Template Quality section. |
| | Poor primer design. Make sure primers are not self-complementary or complementary to each other. |
| | Verify that primers are complementary to the appropriate strands. |
| | Insufficient number of cycles. Return reactions to thermal cycler for 5 more cycles. |
| | Primer concentration was too low. Verify primer concentration in the reaction. Increase primer concentration if necessary. |
| | Suboptimal reaction conditions. Optimize $Mg^{2+}$ concentration, annealing temperature and extension time. Verify that primers are present at equal concentrations. Refer to General Considerations for PCR Optimization for more information about optimizing reaction conditions. |
| | Nucleotides were degraded. Keep nucleotides frozen in aliquots, thaw quickly and keep on ice once thawed. Avoid multiple freeze-thaw cycles. |
| | Target sequence was not present in target DNA or RNA. Redesign experiment, or try other sources of target DNA or RNA |
| | Reaction component was missing. Always perform a positive control reaction with a template/primer combination that has amplified well in the past to determine when a component was omitted. Check the reaction components, and repeat the reaction. |
| | Poor-quality mineral oil. The reaction must be overlaid with high-quality, nuclease-free light mineral oil when using a thermal cycler without a heated lid. Do not use autoclaved mineral oil. |
| | Thermal cycler was programmed incorrectly. Verify that times and temperatures are correct. Use step cycles, not hold segments. |
| | Thermal cycler did not reach the proper temperature. Calibrate the thermal cycler to be sure reactions are heated to the programmed temperatures. Depending on the primers and template, small changes in cycling conditions can affect yield. |
| | Temperature was too low in some positions of thermal cycler. Perform a set of control reactions to determine if certain positions in the thermal cycler give low yields. |

| Symptoms | Solutions |
|---|---|
| Nonspecific amplification products (PCR or RT-PCR) | Reaction conditions were suboptimal. Optimize $Mg^{2+}$ concentration, annealing temperature, primer size, extension time and cycle number to minimize nonspecific priming. Refer to General Considerations for PCR Optimization for more information about optimizing reaction conditions.<br>Perform hot-start PCR to minimize nonspecific amplification. If you are not using a DNA polymerase designed for hot-start PCR, such as GoTaq® Hot Start Polymerase, assemble reactions on ice and preheat the thermal cycler to 95°C before adding reaction tubes.<br>Poor primer design. Make sure primers are not self-complementary or complementary to each other, especially near the 3′-ends. Avoid using three G or C nucleotides in a row at the 3′-end of a primer. Try a longer primer.<br>Primer concentration was too high. Verify primer concentration in the reaction. Try a lower concentration.<br>Reaction was contaminated by another RNA or DNA. Use positive displacement pipettes or aerosol-resistant tips to reduce cross-contamination during pipetting. Use a separate work area and pipettor for pre- and postamplification. Wear gloves, and change them often. Use UNG or another technique to prevent carryover of DNA produced in a previous amplification into subsequent reactions. See the Nucleic Acid Cross-Contamination section.<br>Multiple target sequences exist. Design new primers with higher specificity to target sequence in template DNA or cDNA. |
| Low yield or no first-strand product (RT-PCR) | RNA was degraded. Verify RNA integrity by denaturing agarose gel electrophoresis. Ensure that reagents, tips and tubes are RNase-free. Isolate RNA in the presence of a ribonuclease inhibitor (e.g., Promega RNasin® Ribonuclease Inhibitor). Repurify the DNA or RNA template if the nucleic acid appears degraded.<br>AMV reverse transcriptase was thermally inactivated. If an initial denaturation/annealing step is introduced into the protocol, be certain to add the enzyme mix containing AMV reverse transcriptase after denaturation and subsequent 45°C equilibration.<br>Poor primer specificity. Verify that the reverse transcription primer is complementary to the downstream RNA sequence.<br>Poor primer annealing. If oligo(dT) primers or random hexamers were used as the reverse transcription primer, verify that the annealing step was carried out at an appropriate temperature prior to reverse transcription.<br>RNA template was impure. Carryover of reagents (e.g., SDS, NaCl, heparin, guanidine thiocyanate) from some RNA purification methods can interfere with RT-PCR. Reduce volume of target RNA, perform additional purification steps or change purification method.<br>Target RNA was not present in the sample or was present at low levels. Use poly(A)+ RNA, rather than total RNA, as a template to increase mRNA target abundance. Alternatively, isolate RNA from different starting material with a higher abundance of the desired target RNA |
| Amplification product with a higher-than-expected molecular weight (RT-PCR) | Genomic DNA sequences related to the RNA template contaminated the RNA preparation. Treat the RNA sample with RQ1 RNase-Free DNase to degrade contaminating DNA. |

## IX. References


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