

IX. Troubleshooting

<u>Symptoms</u>	<u>Causes and Comments</u>
Flat amplification curve in the amplification curves window (no apparent amplification)	Template was degraded or of insufficient quantity. Verify the integrity of the DNA or RNA template by electrophoresis. Repeat the DNA or RNA purification if necessary. Add RNasin® Ribonuclease Inhibitor to the reaction to inhibit a broad spectrum of RNases.
	Amplification inhibitor is present in the DNA or RNA template. Reduce the volume of template in the reaction. Repeat the DNA or RNA purification if necessary.
	Add the template in question to the positive control reaction; a significant increase in the C _i value or no amplification in the positive control reaction indicates the presence of inhibitors in the template.
	Be sure that the reactions were assembled correctly (see the Technical Manual supplied with the Plexor™ System).
	Thermal cycler was programmed incorrectly. Verify cycle times and temperatures (Section III, IV or V).
	Data collection settings were incorrect. Data collection must occur during the extension step. The extension time must be sufficient for data collection. Verify the data collection settings.
	The wrong dye or detector was selected, or the dye was incompatible with the instrument. Be sure the selected detectors are appropriate for the fluorescent dyes used.
	The Plexor™ Master Mix may have lost activity. Be sure to store the Plexor™ qPCR and qRT-PCR Systems at -20°C to avoid loss of enzyme activity. Confirm the instrument settings and perform a positive control reaction to determine if there is a problem with the Plexor™ System reagents.
	The primer sequence is incorrect. Verify the primer sequence.
	Poor primer design. Redesign primers, targeting a different region of the gene of interest. We strongly recommend using the Plexor™ Primer Design Software, which is available at: www.promega.com/plexorresources/
Amplification curve shows 2 drops in signal	The passive reference was on. Be sure the passive reference is set at "none" (Figure 1)





IX. Troubleshooting (continued)

Symptoms

Causes and Comments

Flat amplification curve in the amplification curves window (no apparent amplification) (continued)

Primer was degraded. Use MOPS/EDTA Buffer to resuspend and dilute primers. Iso-dC-containing primers are sensitive to pH. Rehydrating or storing the primer in water or a buffer with a pH less than 7.0 will result in primer degradation. Do not use water to resuspend or dilute primers or make primer mixes.

Primers may have been synthesized incorrectly. Resynthesize primers.

Primer concentration was incorrect. Verify the primer concentration by measuring the absorbance at 260nm.

The scale of the Y axis was inappropriate. If the scale of the Y axis is too broad, the change in fluorescence may not be visible. Adjust the scale of the Y axis.

Increasing fluorescence over time

Excessive template was added to the reactions. Dilute the template, and repeat the experiment.

The baseline region was set in a region with significant fluorescence fluctuation. The baseline within the baseline region should be flat. Manually adjust the baseline region (Section X.D.).

The baseline region was set too close to the signal change. Manually adjust the baseline region.(Section X.D.).

Two or more distinct melt curves in the melt curves window

For the Plexor™ qRT-PCR Systems, both RNA and DNA templates can be amplified. Treat the RNA template with RNase-free DNase to eliminate contaminating genomic DNA.

Poor primer specificity. Design new primers with higher specificity to the target. To verify primer specificity, perform a BLAST search with the primer sequence. The primer should not exhibit regions of identity with non-target sequences.

Optimize the annealing temperature. Increase the annealing temperature by increments of 2°C to reduce the synthesis of primer-dimer or nonspecific amplification products.

Pseudogenes or polymorphic genes may exist. Design new primers to avoid regions of identity between gene family members.

Assemble the reactions on ice to minimize the synthesis of primer-dimer or nonspecific product.

Reduce the number of amplification cycles to minimize the synthesis of primer-dimer or nonspecific product.

Check for signal bleedthrough. Calibrate the instrument as instructed by the manufacturer for the dye set used.

Decrease the primer concentration (e.g., 0.1µM).

Primer pairs in a multiplex reaction can interact to form undesired amplification products. Perform a BLAST search to reveal regions of identity with undesirable target sequences. Label the primer with the lowest homology to other sequences. Alternatively, design new primers using the Plexor™ Primer Design Software, which is available at:

www.promega.com/plexorresources/

IX. Troubleshooting (continued)



Symptoms	Causes and Comments
Broad melt curve or a shoulder on the melt curve	<p>Pseudogenes and polymorphic genes may exist. Perform a BLAST search of the target sequence. When designing primers, choose target sequences that have the fewest regions of identity with pseudogenes and polymorphic genes.</p> <p>Check for signal bleedthrough. Calibrate the instrument as instructed by the manufacturer for the dye set used.</p> <p>Decrease the primer concentration (e.g., 0.1μM).</p> <p>Be sure the thermal cycler is programmed correctly (Section III, IV or V)</p>
No melt curve observed in the melt curve window	<p>Poor amplification. See causes and comments for "Flat" amplification curve in the amplification curves window (no apparent amplification)" above.</p> <p>Problems with data export or instrument analysis have occurred. Review the instructions for data export and instrument setup.</p> <p>Data collection settings were incorrect. Verify the thermal cycling program and data collection settings are correct (Section III, IV or V).</p> <p>Instrument was programmed incorrectly. Verify the thermal cycling program is correct (Section III, IV or V).</p>
Variability in signal among replicate samples	<p>Calibrate your pipettes to minimize variability in pipetting.</p> <p>Small volumes are difficult to pipet accurately. Do not pipet volumes <1μl; dilute the template so that larger volumes are pipetted.</p> <p>Some variation is normal. A difference of 1-2 cycles for the C_t values is within the normal variation associated with an exponential amplification reaction.</p> <p>There will be statistical variation in the amount of template in a reaction with targets present at low copy number. Poisson distribution predicts difficulty associated with reliable detection of very dilute samples with few target molecules.</p> <p>Mixing was inadequate. Vortex reagents to mix well prior to pipetting.</p> <p>Plate performance can differ from manufacturer to manufacturer. Use plasticware recommended by the instrument manufacturer.</p> <p>Instrument was improperly calibrated. Calibrate instrument as instructed by the manufacturer.</p> <p>Thermal cycling conditions were suboptimal. Optimize the annealing temperature.</p> <p>Thermal cycling conditions were suboptimal. Redesign your primers, so the melting temperatures are 60°C. We strongly encourage using the Plexor™ Primer Design Software.</p> <p>Viscous samples (e.g., high-molecular-weight genomic DNA) are difficult to pipet accurately. Dilute the DNA template.</p> <p>Shear high-molecular-weight DNA by vortexing or pipetting.</p>



IX. Troubleshooting (continued)

Symptoms	Causes and Comments
Variability in signal among replicate samples (continued)	<p>The baseline region was not set correctly. The baseline should be flat. The baseline region can be adjusted manually for each well to account for sample-to-sample variation (Section X.D.)</p> <p>The plate was not completely sealed. Carefully seal the plates to avoid evaporation.</p>
Fluorescence decrease observed in the no-template control	<p>Nonspecific product can accumulate at higher cycle number in reactions with targets present at low copy numbers. Assemble the reactions on ice to reduce the accumulation of nonspecific amplification products, including primer-dimer.</p> <p>Decrease the cycle number to reduce the accumulation of nonspecific amplification products.</p> <p>Primers designed with another primer-design software or for another amplification. Design new primers using the Plexor™ Primer Design Software.</p> <p>Reactions were contaminated with target DNA or RNA. Clean workstations and pipettes with a mild bleach solution before and after use. Use new reagents and solutions. Take precautions to prevent contamination (see the <i>Plexor™ qPCR System Technical Manual #TM262</i>, the <i>Plexor™ One-Step qRT-PCR System Technical Manual #TM263</i> or the <i>Plexor™ Two-Step qRT-PCR System Technical Manual #TM264</i>).</p> <p>An improperly calibrated instrument can lead to erratic fluorescence readings. Calibrate the instrument as instructed by the manufacturer.</p>
Vertical fluorescence spikes or significant “noise” in the amplification curve	<p>Consult the instrument manufacturer’s user’s guide for information about potential instrument problems that can cause spikes or noise.</p> <p>No amplification or poor amplification for the entire plate. Poor amplification can lead to improper data scaling, making fluorescence measurements appear erratic. See possible causes and comments for “Flat amplification curve in the amplification curves window (no apparent amplification)” above.</p> <p>Instrument was improperly calibrated. Calibrate instrument as instructed by the manufacturer.</p> <p>The passive reference was “On”. Be sure the passive reference is set as [none] (Figure 1).</p>
Small signal change in amplification curve and melt curves	<p>No amplification or poor amplification. See causes and comments for “Flat amplification curve in the amplification curves window (no apparent amplification)” above.</p> <p>Incorrect filter was selected. Verify the presence of the appropriate filter.</p> <p>Primer concentration was incorrect. Verify primer concentration by measuring the absorbance at 260nm.</p> <p>The scale of the Y axis of the amplification curve is affected by other reactions on the plate. A high fluorescent signal for one or more reactions can cause the scale of the Y axis of the amplification curve to be too high to see changes in some data. Adjust the scale of the Y axis to accommodate samples with smaller changes in fluorescence. See Section VIII.C.</p> <p>The passive reference was “On”. Be sure the passive reference is set as [none] (Figure 1).</p>

IX. Troubleshooting (continued)



Symptoms	Causes and Comments
Nonlinear standard curve, low R ² values	<p>An amplification inhibitor is present in the standard reference template. Determine whether the template contains inhibitors by adding the DNA template to the positive control reaction; a significant increase in the C_t value or no amplification of the positive control in the presence of the DNA template indicates the presence of inhibitors. Repeat purification of the standard reference template used to generate the standard curve.</p> <p><u>Calibrate your pipettes to minimize variability in pipetting.</u> Small volumes are difficult to pipet accurately. Do not pipet volumes <1µl; dilute the template so that larger volumes are pipetted.</p> <p>Viscous samples (e.g., high-molecular-weight genomic DNA) are difficult to pipet accurately. Dilute the DNA template.</p> <p><u>Shear high-molecular-weight DNA by vortexing or pipetting.</u> Adjust the baseline region. The baseline region can be manually adjusted for each reaction. See Section X.D.</p> <p>Some variation is normal. Perform duplicate or triplicate reactions for the standard curve to minimize the effect of this variation.</p> <p>There will be statistical variation in the amount of template in a reaction with targets present at low copy number. Perform <u>duplicate or triplicate reactions for the standard curve.</u></p> <p>An error was made during dilution of the standard reference template. Verify all calculations, and repeat dilution of the <u>standard reference template. Do not pipet volumes <1µl.</u> Incorrect concentration values were entered in the Plexor™ Analysis Software. Verify the concentrations for all samples used to generate the standard curve.</p> <p>Reactions were contaminated with target DNA or RNA. Clean workstations and pipettes with a mild bleach solution before and after use. Use new reagents and solutions. Take precautions to prevent contamination (see the <i>Plexor™ qPCR System Technical Manual #TM262</i>, <i>Plexor™ One-Step qRT-PCR System Technical Manual #TM263</i> or <i>Plexor™ Two-Step qRT-PCR System Technical Manual #TM264</i>).</p> <p>The plate was not completely sealed. Carefully seal the plates to avoid evaporation.</p> <p>Aberrant fluorescence can be caused by writing on plates, contamination, fingerprints, etc. Do not write on the plate. Use caution when handling plates. Wear gloves. Do not place plates on surfaces that might be contaminated with a fluorescent material. If you suspect contamination of the benchtop, thermal cycler block or any other area, clean it thoroughly.</p>



IX. Troubleshooting (continued)

Symptoms	Causes and Comments
Slope less than 0.2 (inefficient amplification)	<p>No amplification or poor amplification. See causes and comments for “Flat amplification curve in the amplification curves window (no apparent amplification)” above.</p> <p>Nonspecific amplification can become a problem in later amplification cycles with samples containing small amounts of target template. Decrease the number of amplification cycles.</p> <p>Poor primer design. Design new primers.</p> <p>Annealing temperature is too high. Design new primers with melting temperatures of 60°C. We strongly recommend using the Plexor™ Primer Design Software.</p> <p>Annealing temperature is too high. Optimize the annealing temperature.</p>
Amplification in no-reverse transcription control for the Plexor™ qRT-PCR Systems	<p>Contaminating DNA related to the RNA template is present in the RNA preparation. Treat the RNA template with RNase-free DNase to remove contaminating DNA.</p> <p>Design new primers to span introns to avoid amplification of contaminating genomic DNA.</p> <p>Assemble reactions on ice to minimize the accumulation of nonspecific amplification products, including primer-dimer.</p> <p>Decrease the number of amplification cycles to reduce accumulation of nonspecific amplification products.</p> <p>Design new primers to minimize the synthesis of nonspecific amplification products.</p> <p>Reactions were contaminated with target DNA or RNA. Clean workstations and pipettes with a mild bleach solution before and after use. Use new reagents and solutions. Use positive-displacement pipettes or aerosol-resistant tips to reduce cross-contamination during pipetting. Use a separate work area and pipette for pre- and postamplification. Wear gloves and change them often.</p>
No amplification in the positive control reaction	<p>No amplification or poor amplification. See causes and comments for “Flat amplification curve in the amplification curves window (no apparent amplification)” above.</p> <p>Verify that the thermal cycling program and data collection settings are correct (Sections III, IV or V).</p> <p>Instrument setup problems can cause amplifications to fail. Consult the instrument manufacturer’s user’s guide for more information about potential instrument problems.</p> <p>The Plexor™ Master Mix may have lost activity. Be sure to store the Plexor™ qPCR and qRT-PCR Systems at -20°C to avoid loss of enzyme activity. Confirm the instrument settings, and perform a positive control reaction to determine if there is a problem with the Plexor™ System reagents.</p> <p>The RNA template used in the Plexor™ qRT-PCR System was contaminated with ribonuclease (RNase). Take precautions to prevent RNase contamination. Clean workstations and pipettes with a mild bleach solution before and after use. Use new reagents and solutions.</p>

IX. Troubleshooting (continued)



Symptoms	Causes and Comments
No amplification in the positive control reaction (continued)	<p>The RNA template used in the Plexor™ qRT-PCR Systems was degraded. RNA storage conditions are very important. Store RNA template at -70°C in single-use aliquots to minimize the number of freeze-thaw cycles. Once thawed, keep RNA on ice. Always use nuclease-free, commercially autoclaved reaction tubes, sterile aerosol-resistant tips and gloves to minimize RNase contamination.</p> <p>Reactions were assembled incorrectly. Repeat the experiment and assemble reactions as described in the <i>Plexor™ qPCR System Technical Manual #TM262</i>, <i>Plexor™ One-Step qRT-PCR System Technical Manual #TM263</i> or <i>Plexor™ Two-Step qRT-PCR System Technical Manual #TM264</i>.</p>
Unable to import data. An error like "Expecting NEWLINE, found"" or "Unexpected Token Error" is encountered	The data has been altered after export from the real-time PCR instrument software. Any alteration of this data is likely to change the formatting and can cause import errors. Do not open the exported files with other software programs.
Data display appears abnormal in the Plexor™ Analysis Software (the screen appears compressed, lines are replaced with dots, etc.)	Be sure that the display settings for the computer are set to 32-bit color, rather than 16-bit color, when using the Plexor™ Analysis Software.
Genotyping: Miscalled known heterozygous samples: Product formed with only one of the two genotyping primers	<p>Poor primer design. Redesign primers. We strongly recommend using the Plexor™ Primer Design Software, which is available at: www.promega.com/plexorresources/</p> <p>The annealing temperature is too high or too low. Optimize the annealing temperature.</p>
Genotyping: Miscalled known homozygous samples: Product formed (signal decrease) with both primers	<p>Poor primer design. Redesign your primers. We strongly recommend using the Plexor™ Primer Design Software, which is available at: www.promega.com/plexorresources/</p> <p>The annealing temperature is too low. Optimize the annealing temperature.</p> <p>Reactions were assembled incorrectly. Verify that the appropriate primer pairs were added</p>
Genotyping: Miscalled known homozygous samples: Product formed only with the mismatched primer but not with the matching primer	<p>The primer sequence was incorrect. Verify that the primer sequence is correct.</p> <p>Genotyping primer #1 and primer #2 were switched. Verify that the correct primer was used.</p>
Genotyping: No call	Add more template. Redesign primers. See comments for "Flat amplification curve in the amplification curves window (no apparent amplification)".