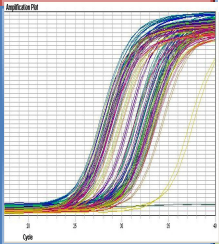
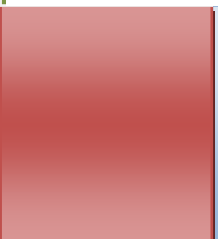


NanoCinna

Pharmacogenomics Center

Real-Time PCR

Troubleshooting guide



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Troubleshooting

Problem	Possible cause	Solution
No amplification	You may have problems setting up the reaction conditions on your real-time PCR instrument.	Follow the specific reagent protocol. Be sure to use the appropriate hot-start temperature to activate the DNA polymerase. Use the appropriate time and/or temperature for amplification.
	Degradation of primers.	Check PCR primers for possible degradation on polyacrylamide gel.
	Pipetting error or missing reagent.	Repeat the PCR reaction; check the concentrations of template and primers; ensure proper storage conditions of all reagents. Make new serial dilutions of template DNA or RNA.
	Primer design is suboptimal.	Verify your primer design, use reputable primer design programs or validated pre-designed primers.
	PCR inhibitors presented in the reaction mixture.	Re-purify your template RNA.
	RT-qPCR: inhibition by excess volume of the RT reaction.	Volume of RT reaction product added to qPCR reaction should not exceed 10% of the total qPCR reaction volume.
	Annealing temperature is not optimal.	Optimize the Annealing temperature in 3 °C increments.
Late amplification	RNA sample may be of poor quality and containing PCR inhibitors.	Perform RNA purification on a sample using a new purification method; Select the extraction method that can assure a high yield and purity of nucleic acid and a complete removal of PCR inhibitors. Further purify your samples- RNA with a significantly lower A_{260}/A_{280}

Late amplification

Reverse transcription may not be optimal and the input amount of RNA may not be optimal for the size of RT reaction

Template sequence may not be enough.

Samples may be containing degraded RNA. Check RNA integrity in denaturing Agarose gel electrophoresis or Agilent 2100 bioanalyzer. Sharp 18S and 28S RNA bands should be visible after denaturing agarose gel electrophoresis of total eukaryotic RNA.

ratio should be further purified by phenol-chloroform extraction, LiCl precipitation, or washing to remove residual salt.

Test your sample using a lower template concentration at which it is known that PCR inhibition does not affect the real-time PCR results.

Check type of reverse transcriptase and priming, quality of starting template, amount and quantification of starting RNA. It is fundamental to accurately quantities the starting template (RNA) to be reverse transcribed.

Gene of interest may not be expressed at a high level in sample type, so that increase the amount of template you use in the real-time PCR reaction.

Select an RNA isolation kit based on your sample type to maximize yield.

Preamplify your target sequences.

Maintain your laboratory workspace free of RNase contamination.

Store samples carefully prior to RNA isolation.

Disrupt samples completely to prevent RNA degradation and to increase RNA yield.

Choose an appropriate RNA isolation kit.

Store RNA in RNase-free solution.

<p>Late amplification</p>	<p>Assay design may not detect all of the splice variants for the gene of interest.</p>	<p>Accurately quantities RNA and confirm that RNA is high integrity and purity.</p> <p>When you use TaqMan® Gene Expression Assay, check the alignment of the assay with target sequence.</p> <p>When you use custom TaqMan® Gene Expression Assay, submit a template sequence that is common to all of the alternative splicing variants.</p> <p>When assay designed by yourself, design the primer and probe on a template sequence that is common for all of the alternative splicing variants.</p>
<p>RT-PCR product longer than expected</p>	<p>RNA template is contaminated with DNA.</p>	<p>Perform DNase I digestion prior reverse transcription. To avoid amplification of genomic DNA, design PCR primers on exon-intron boundaries.</p>
<p>Amplification signal in non-template control</p>	<p>DNA contamination of reagents.</p> <p>RT-qPCR: RNA contaminated with genomic DNA.</p> <p>Primer-dimers.</p>	<p>Follow general guidelines to avoid carry over contamination</p> <p>Discard reagents and repeat with new reagents.</p> <p>Design primers on intron/exon boundaries, treat RNA sample with DNaseI, RNA free prior to reverse transcription.</p> <p>Use melting curve analysis to identify primer-dimers by the lower melting temperature compared to amplicon. If presence of dimers is confirmed:</p> <ul style="list-style-type: none"> • Redesign your primers according to recommendations or use validated pre-designed primers. • Optimize annealing temperature by increasing in 3°C increments.

Poor standard curve	Excessive amount of template.	Do not exceed maximum recommended amounts of template DNA (500 ng DNA for 25 µl reaction).
	Suboptimal amount of template. RT-qPCR: inhibition by excess volume of the RT reaction.	Increase the amount of template, if possible. Volume of RT reaction product added to qPCR reaction should not exceed 10% of the total qPCR reaction volume.
Non-uniform fluorescence intensity	Contamination of the thermal cycler.	Perform decontamination of your real-time cycler according to the supplier's instructions.
	Poor calibration of the thermal cycler.	Perform calibration of the real-time cycler according to the supplier's instructions.

References

1. <http://www.appliedbiosystems.com/absite/us/en/home/support/tutorials/realtime-pcr-trouble-shooting-guide.html>.
2. www.fermentas.com

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