

# Probe QPCR Troubleshooting Guide



## No Amplification

Pipetting error / reagents missing	Repeat experiment checking concentration of all reagents.
Enzyme not activated	Check that the full 15 minute activation step is performed before cycling.
Annealing step	Check that you have the optimal annealing temperature by performing a temperature gradient (2°C increments). Annealing time should be carried out as specified in the protocol.
Extension step	Extension time can be increased for longer amplicons. Amplification of products over 300bp is not recommended.
Primers	<b>Poor primer design</b> – Check for primer dimers on gel. <b>Wrong primer concentration</b> – 0.4µM recommended. <b>Primers degraded</b> – Check on polyacrylamide gel. Re-order new primers if necessary.
Detection step	Detection reading taken at wrong step. Reading should be taken in the annealing step for Molecular Beacons or the detection/annealing step for hybridisation probes.
Product too long	The ideal amplicon size is between 80–200bp. Amplification of products over 300bp is not recommended.
Too few cycles	40 cycles is recommended.
Template	<b>Impure template</b> – Purify template before use. For templates isolated from difficult sources (such as plant) use a commercial mix containing enhancers/detergents. <b>Wrong concentration</b> – A concentration of up to 500ng can usually be used. <b>Degraded</b> – Make fresh dilution from stock. Check storage conditions.
Wrong dye layer	Check that machine settings correspond with experiment.
Probes	<b>Poor probe synthesis</b> – Check with rDNase I digestion. Once digested the fluorescence should increase greatly as the quencher and fluorophore become separated. <b>Poor probe design</b> – See “Designing Dual Labelled Probes” <a href="http://www.abgene.com/downloads/Guide_QPCR-probedesign.pdf">http://www.abgene.com/downloads/Guide_QPCR-probedesign.pdf</a>

## Wavy/Erratic Lines

No ROX	Check machine settings. ABI PRISM® requires ROX for normalisation. ROX is available included in ABsolute™ mixes or as a separate vial.
Too many cycles performed	Reduce number of cycles.
Wrong detection step	Check detection step is set in the correct stage of the cycle.
Machine needs calibrating	Wavy lines can be caused by mirror misalignment or lamp problems. Consult machine manufacturer.
Baseline for ΔRn set at wrong cycle	ΔRn should be set between 3-15 cycles and at least 2 cycles before 1 <sup>st</sup> dilution amplifies.
Reaction volume too low.	Some QPCR instruments (e.g ABI® PRISM 7700) are set to read accurately only at volumes of at least 15µl.

## Amplification in No Template Control

Primer dimers	Primer dimers can also be identified by using a serial dilution of your template and running products on a gel. As template concentration increases, the primer dimer bands should decrease in intensity. If the presence of primer dimers is observed, then it may help to do one of the following: 1) re-design the primers, 2) try increasing the annealing temperature, or 3) decrease the primer concentration. If using a standard curve, a sub-optimal gradient can indicate inhibition from primer dimers.
Contamination	<p><b>Template contaminated</b> - Purify template before use. If doing QRT-PCR, treat RNA template with recombinant DNase I or design exon-spanning primers.</p> <p><b>DNA polymerase contaminated</b> – All recombinant DNA polymerases will contain small amounts of <i>E.coli</i> DNA. Thermo-Start® contains minimal amounts compared to competitor enzymes. However, if contamination remains a problem, a 'BLAST' search can be performed to check for homology to the <i>E.coli</i> genome.</p> <p><b>Reagents contaminated</b> – Repeat with fresh reagents and always use filter tips.</p>

## High Well-to-Well Variance

Poor plate choice	Do not use frosted or black plates.
Low quality sealing material	Use only high quality optically clear seals that have been specifically designed for fluorescence applications.
Machine needs re-calibrating	Follow manufacturer's guidelines.
Evaporation	Do not use corner wells or use a more robust seal.
Concentration gradient formed in vial	Invert the mixture a couple of times before use.

## Low Sensitivity (High Cycle Threshold (Ct))

Evaporation	Do not use corner wells or use more robust seal.
Low quality sealing material	Use only high quality optically clear seals that have been specifically designed for fluorescence applications.
Primer dimers	Primer dimers can also be identified by using a serial dilution of your template and running products on a gel. As template concentration increases, the primer dimer bands should decrease in intensity. If the presence of primer dimers is observed, then it may help to do one of the following: 1) re-design the primers, 2) try increasing the annealing temperature, or 3) decrease the primer concentration. If using a standard curve, a sub-optimal gradient can indicate inhibition from primer dimers.
dUTP	dUTP is not as efficient a substrate as dTTP (Longo <i>et al</i> , Gene 1993). This reduction in PCR efficiency will reduce QPCR sensitivity, resulting in higher Ct values. ABgene® recommend the use of dTTP for most applications.
Annealing step	Check that you have the optimal annealing temperature by performing a temperature gradient (2°C increments). Annealing time should be carried out as specified in the protocol.
Extension step	Extension time can be increased for longer amplicons. Amplification of products over 300bp is not recommended.
Primers	Wrong primer concentration – 0.4µM recommended.

Longo *et al* (1990). Use of uracil DNA glycosylase to control carry-over contamination in polymerase chain reactions. *Gene* **93**: 125–128.