qPCR Troubleshooting

Sometimes, no matter what precautions are taken, experiments don't go as planned. At Azure Biosystems, we embrace the challenges of qPCR. Check out these 5 common qPCR issues. Here, we explore how they happen and provide insight into solutions that we've learned through experience.

Problem	Possible causes	Solution
Poor reaction efficiency	A common cause of poor reaction efficiency is the **presence of PCR inhibitors**. If poor efficiency AND an R^2 value of < 0.98 are observed, likely culprits are pipetting error or the standard curve was not prepared fresh.	PCR inhibitors can be diluted away from a sample. Therefore, **dilute the template** prior to generation of the standard curve to find an ideal Ct range specific to your primer pair. To become proficient at pipetting, prepare each sample in technical triplicate, and if using a multichannel pipette, verify by eye that each sample is drawn up identically. Furthermore, ensure that a standard curve/dilution series is prepared fresh, as stored samples can evaporate over time.
Unexpected values	Particularly when using a shared instrument, **other users may have made changes to an existing protocol**. Other potential causes are incorrectly labeled samples or incorrect detection/ selection of wells prior to the qPCR run.	**Check your thermal cycling conditions before the run** to ensure the existing protocol is correct. Verify that the correct dyes, volume, and wells are selected for detection by the instrument. Setup a specific user account for each lab or group using the instrument to store saved protocols.
Inconsistency amongst biological replicates	Inconsistency from sample to sample could be a sign of **RNA degradation or minimal starting material**.	Prior to reverse transcription, check your RNA concentration and quality with a spectrophotometer and/or run your RNA out on an agarose gel. Anything less than the ideal 260/280 ratio of 1.9–2.0 could indicate the presence of PCR inhibitors, and a smear instead of two (2:1) bands on a gel indicates degradation. **Repeat the RNA isolation again and consider using a method more suited to your needs, e.g. silica spin column vs phenol-chloroform.**
Ct values are too early	Solving this issue requires some research into your existing primer pair. The primers may not span an exon-exon junction or could be generating more than one product. **The transcript might be naturally highly expressed** in your samples. If the samples have been stored for some time, evaporation may have caused an increase in the concentration.	By ensuring your primers span an exon-exon junction, the chance for genomic DNA contamination is minimized. However as a preventative step, it is best to DNase treat the samples prior to reverse transcription. To verify only one product is produced, BLAST the primers to determine hits within the organism, include a melt curve at the end of thermal cycling, and/or run the qPCR products out on a gel. If the transcript is naturally highly expressed, **use the same dilution factor across all samples to dilute the template, yielding an ideal Ct range**. To prevent evaporation from samples, ensure tube caps are sealed with parafilm for long-term storage.
Amplification in the no template control (NTC)	When pipetting template into wells that already contain a master mix, the template can sometimes **splash into an adjacent (NTC) well**. Detection is particularly likely if the primers target a highly expressed, well-conserved region. Additional causes include reagent contamination or primer-dimer formation.	Clean your work area and pipettes with 70% ethanol, using 10% bleach if reagents have spilled. **Prepare a fresh primer dilution and be extremely cautious when pipetting the template** (to prevent splashing). Separate the NTC from any template samples on the plate as best as possible and use new reagents if necessary. To detect primer-dimer formation, add a dissociation curve (melt curve) at the end of qPCR cycling and look for the presence of an additional peak, typically at a lower T_m.

www.biozym.com - support@biozym.com

www.azurebiosystems.com • info@azurebiosystems.com