

QPCR Optimization

Optimization Parameter	Recommendation
QPCR Plate	It is recommended that opaque white PCR plates are used for QPCR analysis. The white color eliminates cross-talk and improves the efficiency of fluorescent detection thereby increasing assay sensitivity and well-to-well consistency.
Template Quality	It is essential that the nucleic acid is sufficiently pure for QPCR analysis. Template contamination (ie. genomic DNA, protein, carbohydrates or organic solvents) can have a huge impact on assay reliability and reproducibility. Template quality should be determined by spectrophotometry (ie. Nanodrop), microfluidics or PAGE.
Amplicon Size	Ideally, the amplicon should always be between 100bp and 150bp to ensure the QPCR reaction efficiency is as close to 100% as possible. Good QPCR efficiency promotes assay reproducibility and sensitivity.
Primer Design	Given that PCR primers are a relatively cheap component of a QPCR assay, it is good practice to order and test at least 2 primer pairs for every new QPCR assay. This will maximize the chance of establishing a reliable, reproducible and sensitive assay.
Test Primers	Measure the reproducibility, specificity, sensitivity and dynamic range of your QPCR assay using SYBR Green chemistry across a template dilution series. Ideally, the efficiency of the QPCR reaction should be at least 90% and below 105%, while the assay reproducibility should be higher than $r=0.998$.
Efficient RT	Initially, the RT step should be performed as specified in the supplier protocol. However, the length and the temperature of the RT step can be optimized to increase the efficiency of the reverse transcriptase. The reverse transcriptase should be tested across a range of RNA concentrations to ensure assay linearity.
Hot-Start	All standard Thermo Scientific QPCR master mixes require a 15 minute heating step at 95°C to ensure the hot-start <i>Taq</i> is fully activated. This step cannot be shortened under any circumstances. A shorter heating step will impact assay reproducibility and sensitivity.
Thermal Protocol	Even if the assay has been optimized using an alternative master mix, it is advisable to start by using the thermal protocol recommended in the Thermo Scientific QPCR master mix protocol. If assay optimization is required, the annealing temperature should be examined first.
Annealing Temperature	Test a range of annealing temperatures. Depending on the QPCR results the annealing temperature should be increased or decreased in 2-3°C increments. This can be done in a single experiment using a thermal gradient. Alternatively, a range of annealing temperatures should be tested using multiple QPCR experiments.
Primer Concentration	Always start by using the primer concentration recommended in the master mix protocol. If optimization is required, try stepping the primer concentration up and down in 25mM increments. Optimizing primer concentration using a titration matrix can give improved results in rare circumstances but this is time-consuming.