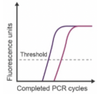
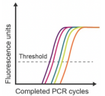
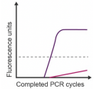
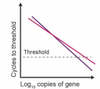
Observation	Cq is Earlier than Expected	Variable Technical Replicates which have Cq Difference of >0.5 Cycles	Unexpected Data Values	No Data in Wells Selected for Analysis	Exponential Amplification of the No Template Control	Plateau is Lower than Expected	Comparisons between sample are Irreproducible
							
Potential Causes	• The RNA has been contaminated by genome DNA • There are multiple products • There is a 'multicopy' gene • Poor specificity of primer • High primer-dimer production when using binding dye detection • Transcript has high expression within the experimental samples	• Pipetting error • Insufficient mixing of the solutions • Low expression of target transcription resulting in stochastic amplification • Reaction is poorly optimised • There is a high Cq and low concentrations of the template	• Samples labelled incorrectly • Plate has been inserted backwards • Poor primer specificity • Inhibitors in the sample	• Wells were not selected for analysis • The wrong dye is selected for analysis • First-strand synthesis failure • PCR failure • No expression of the target transcript	• There is contamination from laboratory exposure to the same target sequence • There is contamination carried over from the reagent manufacture	• Reagents are limiting • Reagents are degraded (dNTPs or master mix) • Some probe dyes are less bright than others • The reaction is inefficient • Probe concentration is incorrect	• The amplification efficiency is below 88% in one or both samples • Differences in efficiency are >5% • Degradation of RNA • Dilutions are inaccurate • Low concentrations are being measured
Corrective Steps	• The specificity of the primers require re-designing to be improved • The assay performance needs to be tested carefully against quantified controls • The primer concentration should be optimised • The annealing temperature should be optimised • DNase treatment should be used before reverse transcriptase	• Pipettes should be calibrated • Use positive-displacement pipettes and filtered tips • Mix all solutions thoroughly during preparation • Hold pipette vertically when aspirating solutions - sterile technique does not ensure reproducibility when working with small volumes • Optimise reaction conditions • Add more sample to achieve lower Cq	• Rerun samples or plate with extra caution when loading • Primers are re-designed to increase specificity • Run a dilution of samples. Inhibitors will be diluted and may result in lower Cq for diluted material	• Settings for data collection and data viewing should be checked and reviewed • Determine whether background fluorescence can be seen • Repeat experiment with new reagents • The assay performance should be tested against carefully quantified controls • Check samples with an alternative target	• Work area should be cleaned with 10% bleach and nuclease-free water • Prepare the reaction mix in a clean lab, separated from any template sources • New reagent stocks should be ordered • Ensure reagents are not contaminated by bacteria when using bacterial templates	• Calculations should be checked for master mix • Repeat experiment using fresh stock solutions • Compare end-point fluorescence to a different reaction using a probe labelled with the same dye	• Redesign primers for one or both genes • Repeat experiment with fresh reagents and sample • Check samples with a target that is more highly expressed