Guidelines for testing PCR Biosystems qPCR products

We recommend you carry out these tests whenever you use one of our qPCR products or product samples for the first time. Following these recommendations will ensure the validity of your qPCR data and ensure the most accurate comparisons.

1.1 General comments

Our qPCR mixes are available in multiple variants, with key products listed in Table 1. Please note, this guide also applies to all PCR Biosystems qPCR products, not only those included in the table below. Products should be chosen based on the planned experiments and in accordance with the passive reference dye requirements of the qPCR instrument(s) to be used. If you are uncertain of which mix is appropriate for your instrument, use our qPCR Selection Tool to find compatible mixes, or contact technical@pcrbio.com with any further questions.

Table 1: Key PCR Biosystems qPCR mixes*.

qPCRBIO SyGreen® Mix	qPCRBIO SyGreen® Mix Blue
Lo-ROX	Lo-ROX
Hi-ROX	Hi-ROX
Separate-ROX	Separate-ROX
Fluorescein	Fluorescein
qPCRBIO Probe Mix	qPCRBIO Probe Blue MIx
Lo-ROX	Lo-ROX
Hi-ROX	Hi-ROX
No-ROX	No-ROX
Separate-ROX	Separate-ROX
Clara® Probe (Purple) Mix	Clara® Probe (1-Step) Purple Mix
Lo-ROX	Lo-ROX
Hi-ROX	Hi-ROX
No-ROX	No-ROX
Separate-ROX	Separate-ROX

*Please note, this guide applies to all PCR Biosystems qPCR and 1-step RT-qPCR mixes, not just those listed in this table.

1.2 Setting up test experiments

When setting up an experiment or comparing one of our mixes to competitor products, all primers, probes, template, diluent, and any other additives used to prepare reactions should originate from the same batch and preferably bottle. All reactions should be prepared on the same day, ideally by the same person. The same instrument and, if available, a liquid handling robot must be used for all comparisons.

Where absolute sensitivity is to be compared between the mixes, genomic DNA should be used. However, care must be taken not to over-interpret the results, because at low template dilutions stochastic effects take over. How to deal with this scenario is discussed in the Troubleshooting section of our qPCR Technical Guide.

Test experiments should be set up as follows:

- Test samples should include 4-5 template dilutions and a no template control (NTC).
- If reverse transcription reactions are carried out independently of the qPCR, *i.e.*, you are not using a 1-step qPCR product, you should also include a no reverse transcription control that contains a true sample and all the same reagents as a regular sample minus reverse transcriptase.
- Set up 3-4 technical replicates for each test and control sample.
- Template dilutions should be selected such that they cover a broad range, and all can contribute to a standard curve (a standard curve should at the very least consist of four points, see Table 2).
- Each reaction volume should be proportional to the vessel used (*e.g.*, at least 20 µL for a 96-well plate) and the plate/tubes should be kept at 4 ˚C throughout the setup. Ideally, mastermixes as well (though these can be set up at room temperature since all polymerases are hot start).

Table 2: Recommended experimental setup for qPCR product testing

Sample	Dilution 1	Dilution 2	Dilution 3	Dilution 4	No template control (NTC)	*No Reverse transcriptase control (NRT)
Content	*e.g.*, 10^{0} undiluted reference sample	*e.g.*, 10^{-2} dilution of Dilution 1	*e.g.*, 10^{-4} dilution of Dilution 1	*e.g.*, 10^{-8} dilution of Dilution 1	Sterile water as sample	Contains all reagents and sample but not reverse transcriptase
Replicates	x3-4	x3-4	x3-4	x3-4	Dilution x3-4 1	x3-4

*Necessary if you will be carrying out a separate cDNA synthesis step independently of qPCR, therefore it does not apply to qPCRBIO 1-Step mixes.

Table 2 summarises the recommended test and control samples you should use when testing our products.

Care must be taken to avoid any type of contamination, particularly with samples that contain the target template at high concentrations, as these are often reported to contaminate NTC reactions. Use a clean bench or dedicated qPCR space for plate setup. The PCR plate must be sealed properly, *i.e.*, ensure full sealing of each individual well, and spin down in a microplate centrifuge to remove all air bubbles.

1.3 Cycling

Before running the comparison, cycling conditions must be optimised following each manufacturer's guidelines, to ensure that every mix performs at the best of its abilities for a given template-primer set.

If comparing different manufacturer's mixes in the same run, switch the passive reference off when testing on an instrument requiring ROX or Fluorescein.

If the optimised cycling conditions differ between the mixes, it is advisable to run each mix at its optimal conditions, as well as each of the other competitors' conditions.

The same number of cycles must be used in the case of separate runs.

Each run should be followed by an identical melt curve analysis.

The plate should be examined after each run to make sure that it was sealed properly and that there was no reaction mix evaporation.

1.4 Data analysis

All wells showing spurious signal, or compromised reaction specificity, should be excluded from the analysis.

Differences in fluorescence intensity levels among different supplier's mixes are of little importance, provided they are within the same order of magnitude, unless a good rationale for such differences exists. The amount of dynamic range inherent in qPCR mixes is more than sufficient to explain such differences.

Direct comparison of different mixes poses a problem for threshold C_q estimation methods. Due to different fluorescence levels of different mixes, the threshold method can underestimate C_q values for high fluorescence level mixes. This can be mitigated by normalisation of the signal (essentially matching all plateaus). Please compare the raw signal to the output to determine whether your software performs such normalisation by default. If your software allows for C_q estimation by extracting the C_q parameter from curve fitting or if you can export the data and perform a custom-made analysis, then this will fix false differences between C_q values that arise from differences in fluorescence level. Otherwise, C_q values can be assigned by visualisation and moving the C_q threshold where possible with some software (*e.g.*, LinRegPCR).

A standard curve should be calculated for each dilution series. Slope (efficiency) as well as intercept of the curve provide information on the quality of the PCR amplification. Efficiency of 2 (or 100%) is ideal, meaning that the amount of DNA doubled between each cycle. Efficiencies higher than that likely indicate error in the setup or the presence of inhibitors in the samples. Lower efficiencies suggest that the reaction conditions are not optimal: incorrect buffer, bad primer design, the wrong amount of dNTPs, Mg^{2+}

or enzyme, or one of the components are degraded. Intercept provides a measure of the sensitivity of the assay. A lower value corresponds to higher sensitivity. However, this is a relative measure that must only be used when comparing the same reaction setup (same amplicon, same cycling conditions, same instrument). Please note that we do not advise comparing C_q values generated with different supplier mixes at a single template dilution since the reactions may perform at different efficiencies.

The melt curves should be analysed for the presence of primer-dimers. The presence of a melt peak at a temperature lower than that of the PCR product indicates the formation of primer dimers. This is particularly visible at lower temperature dilutions and indicates poor hot start activity. Multiple peaks likely result from poorly designed primers or non-specific priming from inefficient hot start approaches. Peaks higher than that of the PCR product could indicate genomic contamination. The absolute position of the peaks often varies between different mixes manufacturers due to variations in buffer composition and should not be a source of concern.