10 tips to maximize your real-time PCR success

Sample to Insight

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Improve your real-time PCR

1) Use a high-quality nucleic acid template

PCR consists of multiple rounds of enzymatic reactions. Therefore, it is more sensitive to impurities (e.g., proteins, phenol, chloroform, salts, and EDTA) than single-step, enzyme-catalyzed reactions. Your nucleic acid templates should be pure: this is particularly important for real-time PCR because contaminants can interfere with fluorescence detection. Commercial suppliers offer a wide range of nucleic acid purification systems that yield pure, high-quality templates.

2) Determine the concentration and purity of your template

Check the integrity and size distribution of your purified total RNA using denaturing agarose gel electrophoresis and ethidium bromide staining or a capillary electrophoresis system. The ribosomal RNAs (rRNA) should appear as sharp bands or peaks. The apparent ratio of 28S rRNA to 18S rRNA should be approximately 2:1. If the ribosomal bands or peaks of a specific sample are not sharp, but rather appear as a smear towards smaller sized RNAs, it is likely that the sample suffered major degradation either before or during RNA purification.

Determine the concentration of your purified DNA or RNA template by measuring the absorbance at 260 nm (A_{260}) in a spectrophotometer. Absorbance readings at 260 nm should fall between 0.15 and 1.0. Note that absorbance measurements at 260 nm cannot discriminate between DNA and RNA. Depending on the method used for template preparation, the purified DNA may be contaminated with RNA, or the purified RNA may be contaminated with DNA. If this is the case, the A_{260} value will be too high, leading to inaccurate quantification.

The ratio between the absorbance values at 260 nm and 280 nm (A_{260}/A_{280}) gives an estimate of the purity of the DNA or RNA template. As this ratio is considerably influenced by pH, the absorbance measurements should be made in 10 mM Tris-Cl at pH 7.5. In this buffer, pure DNA has an A_{260}/A_{280} ratio of 1.8–2.0, and pure RNA has an A_{260}/A_{280} ratio

of 1.9–2.1, although values of up to 2.3 are routinely obtained for pure RNA with some spectrophotometers. Lower ratios indicate the presence of contaminants, such as proteins.

3) Check your nucleic acid storage conditions

Store purified DNA at –20°C or –70°C under slightly basic conditions (e.g., Tris-Cl at pH 8.0 or a dedicated storage buffer from a commercial supplier), as acidic conditions can cause depurination of DNA. Store purified RNA at –20°C or –70°C in RNase-free water. It's best to store diluted solutions of nucleic acids (e.g., dilution series used as standards) in aliquots. If one of the tubes is accidentally contaminated, you can use a fresh one without worrying about contaminating the main stock. We recommend storing aliquots in dedicated tubes that prevent adherence of nucleic acids to the tube walls. Otherwise, the concentration of nucleic acids in solution may be reduced. Finally, it is important to thaw the aliquots once only. Repeated freezing and thawing damages the nucleic acids.

4) Use the optimal template amount

Even for low-abundance targets, we recommend using no more than 100 ng of template RNA or cDNA. Generally, 1–100 ng of template will be sufficient. With the appropriate qPCR kit, you can detect as little as one target copy corresponding to 3 pg of DNA. Template purity is important if you are adding large volumes of low concentration template to the reaction. Using highly pure DNA or RNA, the template can contribute up to 40% of your final reaction volume as long as you don't exceed the recommended template amounts. If you use cDNA from an RT reaction as your template, don't let the volume of undiluted RT reaction exceed 10% of the final PCR volume.

5) Determine reaction efficiency for every new primer pair

The amplification efficiencies of the various qPCR setups generally differ because the efficiency of primer annealing, the GC content of the sequences to be amplified, and the qPCR product sizes generally vary for the two genes. The quantification of the targets differs depending on whether the two targets are amplified with the same reaction efficiency. To ensure accurate quantification, you should determine the reaction efficiency for every qPCR assay by preparing a standard curve.

You can compare the amplification efficiency of 2 genes (targets A and B) by preparing a dilution series for both genes from a reference RNA or cDNA sample. Then, you amplify each dilution series in real-time one-step or two-step RT-PCR. Use the resulting C_T values to construct standard curves for targets A and B. You can calculate the amplification efficiency (E) for each target according to the following equation:

$$E = 10^{(-1/S)} - 1$$

where S is the slope of the standard curve.

6) Store primers and probes correctly

For optimal results in real-time PCR and RT-PCR, obtain your primers and probes from an established oligonucleotide manufacturer. Resuspend the lyophilized primers and probes upon receipt, and check their concentrations using spectrophotometry. Dissolve the lyophilized primers and probes in TE buffer consisting of 10 mM Tris·Cl and 1 mM EDTA at pH 8.0 to provide a 100 µM stock solution, and divide this into small aliquots so you can avoid repeated freezing and thawing. We do not recommend storing primers at concentrations below 10 µM. Keep reconstituted primers at –20°C and monitor them for signs of decreased functionality if stored for more than a year. Protect labeled primers and probes from light.

7) Prevent contamination

You should minimize the potential for sample cross-contamination and nucleic acid carryover from one experiment to the next. Use aerosol-resistant pipette tips. Work in designated, separate areas and use separate pipettes for pre and post-amplification steps. Wear gloves and change them often. Don't open reaction tubes after amplification is complete, because opening increases the risk of contaminating subsequent reaction setups with the amplified product.

8) Mix all of your reaction components well

Before assembling your qPCR reaction, briefly mix, and then centrifuge all of your reaction components. Gently swirl the enzyme-containing master mix or invert the master mix tube a few times and briefly vortex other components, such as template nucleic acid, and primer and probe solutions. However, you have to avoid over-vortexing, because it can cause bubbles to form, which can interfere with fluorescence detection and reduce enzymatic activity. Always centrifuge all of your reaction components briefly after mixing to collect the contents at the bottom of the vessel and eliminate any air bubbles from the solution.

9) Perform control reactions

In real-time PCR, controls prove that the signal obtained from the experimental samples represents the amplicon of interest. All qPCR experiments should include a no-template control, and one-step reactions should also include a no-RT control.

No-template controls

A no-template control (NTC) allows you to detect contamination of the PCR reagents or primer-dimers. An NTC reaction contains all of the real-time PCR components except the template. Detection of fluorescence in an NTC reaction indicates the presence of contaminating nucleic acids or primer dimerization.

No-RT control

A no-RT control involves real-time RT-PCR carried out without reverse transcriptase. You should always include one when performing gene expression analysis. For viral load monitoring, a no-RT control may be necessary, depending on the sample type and the life cycle of the virus species detected. Since reverse transcription cannot take place, a no-RT control reaction allows you to detect contaminating DNA, such as DNA from viral sequences integrated into the host genome. Remove contaminating DNA with DNase treatment before you start your one-step RT-PCR.

Internal positive control

An internal positive control tests for the presence of PCR inhibitors. If you carry out a duplex reaction where the target sequence is amplified with one primer–probe set, the control sequence (i.e., the internal positive control) is amplified with a different primer–probe set. The internal positive control should be at a high enough copy number for accurate detection. If the internal positive control is detected, but the target sequence is not, then this indicates that the amplification reaction was successful and that the target sequence is absent (or present at too low a copy number to be detected). If the internal positive control is not detected, then amplification failed, most likely due to the presence of PCR inhibitors.

Positive control

You may need a positive control, for example, when you're amplifying a new target sequence and you want to confirm that the primer set or primer–probe set works. A positive control can be an absolute standard, which is a nucleic acid template of known copy number that provides quantitative information. Absolute standards include nucleic acids from established cell lines, plasmids containing cloned sequences, and in vitro-transcribed RNA, and they are commercially available or can be generated in the lab. A positive control can also be a known positive sample, which is usually a substitute for an absolute standard and used only to test for the presence or absence of a target.

10) Double-check your cycler settings before your run

Checking your cycler settings is especially important if you're using a shared instrument. Even if you set up your own template file, ensure that the machine has the correct run cycle for your experiment. Some instruments default back to their standard settings so you may find that your settings didn't save. Ensure that the correct detection channel is activated or the correct filter set is selected for the dyes you are using.

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