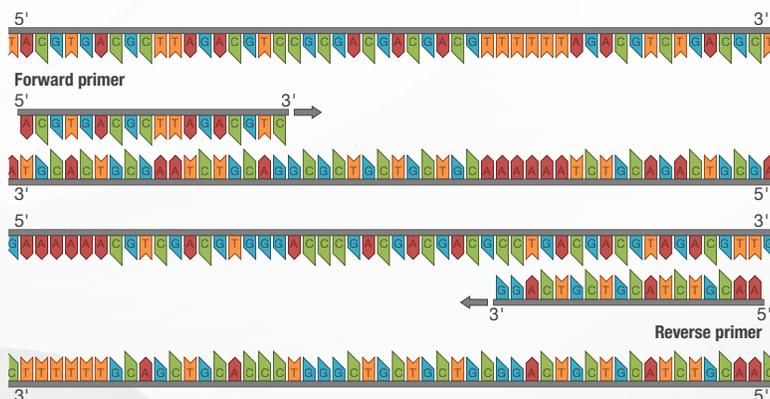


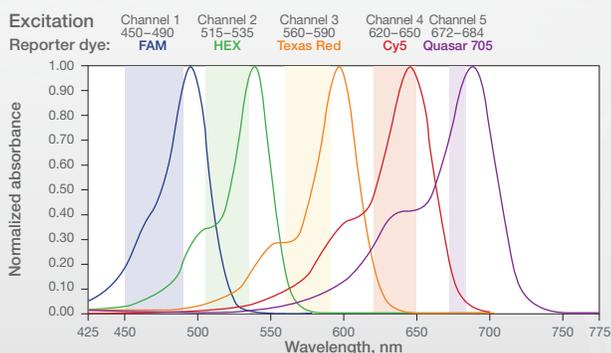
# The Ultimate qPCR Assay Design Guide

## 8 TIPS FOR DESIGNING PRIMERS

1. Design amplicons that are 70–150 bp long
2. Design primers that have a GC content of 40–60%
3. Avoid sequences with long (4+) repeats of a single base
4. Make sure your primers have a melting temperature between 50 and 65°C
5. Avoid designing primers for regions with secondary structures. Use programs like MFOLD to map predicted structure of your target sequence
6. Design your primers to span an exon-exon junction if you are assessing gene expression in eukaryotic cells to avoid amplifying any contaminating genomic DNA that may carry over from the purification step
7. Check the sequences of the forward and reverse primers for 3' complementarity. This can result in primer-dimer formation
8. Verify specificity by using any online tool, such as Primer-BLAST, to confirm the target of interest is unique



## 5 TIPS FOR DESIGNING HYDROLYSIS PROBES



1. Design probes with melting temperatures 8–10°C higher than that of the primers
2. Probes should be shorter than 30 nucleotides, for most applications. If the probe is longer, consider using an internal quencher
3. Make sure your probe does not have a G at its 5' end. 5' G's can quench fluorescence even after hydrolysis
4. Design your probe, or one of your primers, to span an exon-exon junction if you are assessing gene expression in eukaryotic cells
5. Run the probe sequence through a Primer-BLAST alignment to ensure the sequence is unique to your target of interest

## 6 TRICKS FOR INCREASING REPRODUCIBILITY

1. Follow best practice guidelines for minimizing contamination
2. Make sure your pipets are properly calibrated
3. Use a no template control to verify the absence of contamination
4. Prepare enough master mix to run all your reactions + 10% extra
5. When preparing technical replicates, add the template to the master mix and not to individual reactions
6. Avoid pipetting less than 5 µl



## 7 LABORATORY BEST PRACTICES TO MINIMIZE CONTAMINATION

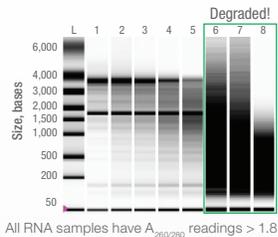


1. Wear gloves and work in a dedicated qPCR area
2. Use screwcap tubes for template
3. Always use dedicated pipets for qPCR
4. Use aerosol-resistant filter tips
5. Use PCR-grade water
6. Clean bench with 10% bleach, not ethanol! Ethanol only precipitates DNA and spreads it around on surfaces
7. Always include a no template control

## TIPS, TRICKS & BEST PRACTICES

# 9 Potential Real-Time PCR Pitfalls (and How to Avoid Them!)

### 1 USING DEGRADED RNA



When it comes to qPCR, garbage in = garbage out. Degraded or contaminated RNA will yield low-quality cDNA, which will produce poor qPCR reaction efficiencies and result in low-quality data that are analytically inaccurate.

#### How to avoid

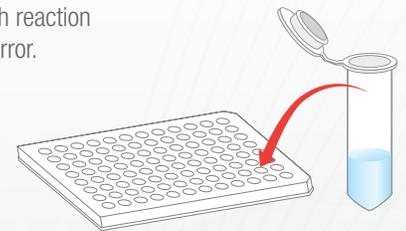
Assess the quality of your RNA by gel electrophoresis or by bioanalyzer analysis

### 2 PIPETTING REACTION COMPONENTS SEPARATELY

qPCR reaction efficiency is highly dependent on the chemical composition in the reaction vessel, thus each reaction must occur in an identical chemical environment. Pipetting components separately invites variability and error.

#### How to avoid

- Prepare a master mix that contains all reaction components except template, mix thoroughly, and dispense into each well
- Prepare enough master mix to run all of your reactions plus 10% extra to compensate for potential pipetting errors. Do not freeze and reuse



### 3 INTRODUCING CROSS-CONTAMINATION



Amplified DNA is easily aerosolized if caution is not used when handling tubes. Contamination introduced into a reaction vessel or reagent will skew your data, producing biologically irrelevant results.

#### How to avoid

- Wear gloves and work in a dedicated qPCR area
- Use screwcap tubes for template
- Always use dedicated pipets for qPCR
- Use aerosol-resistant filter tips
- Use PCR-grade water
- Aliquot PCR components for single-time use
- Clean bench with 10% bleach, not ethanol!
- Always include a no template control

### 4 FORGETTING TO INCLUDE CONTROLS

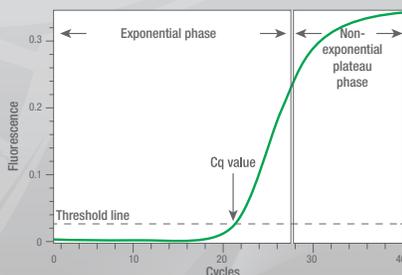
How do you know if your target sequence amplified correctly? Without controls, you will not be able to speak to the specificity and accuracy of the assay or troubleshoot if something goes wrong.

#### How to avoid

- **No template control:** Omit sample from 1 well to serve as a control for extraneous nucleic acid contamination
- **No reverse transcription (RT) control:** For each assay include 1 well that uses a no reverse transcriptase cDNA sample as the template
- **RNA quality control:** Use an RNA quality assay to verify RNA integrity
- **Positive and negative controls:** Add a synthetic template to a reaction to demonstrate the reaction conditions are correct. Omit DNA polymerase from a negative control to assess background fluorescence signal



### 5 SETTING THE THRESHOLD TOO HIGH OR TOO LOW



If the threshold is set outside the exponential growth phase of the reaction, the Cq value will not accurately reflect the DNA concentration of the sample and results will be biologically irrelevant.

#### How to avoid

- If the plate includes serial dilutions, adjust the threshold to a position where you reach a maximum correlation coefficient ( $R^2$  value) for the standards
- If the plate does not contain serial dilutions, place the threshold in the exponential growth phase, above the noise but below the plateau phase



## 6 FAILING TO OPTIMIZE AND VALIDATE AN ASSAY

To achieve accurate template quantification in a qPCR assay, each reaction must efficiently amplify a single product. Amplification efficiency must be independent of:

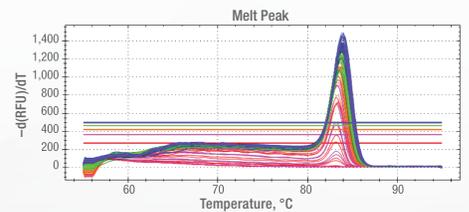
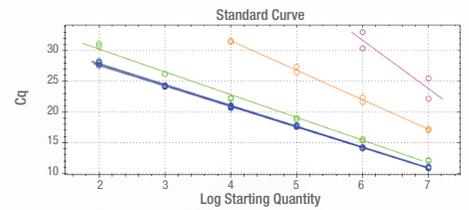
- Template concentration
- Amplification of other templates
- Potentially contaminating compounds in the sample

Even commercial assays should be validated under your specific and unique reaction conditions.

### How to avoid

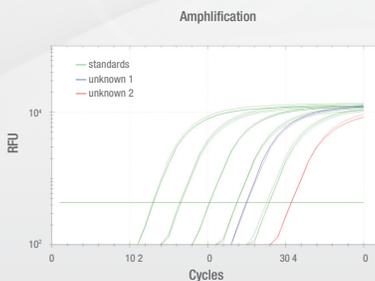
Always validate a new qPCR assay to verify its efficiency under your specific conditions

- Determine efficiency using a standard curve spanning 5 orders of magnitude (5- or 10-fold dilutions) and run in triplicate to determine the efficiency, linear dynamic range, and reproducibility of the assay
  - ◆ Efficiency of the PCR should be 90–110%
  - ◆  $R^2$  of the standard curve should be  $> 0.98$
  - ◆ Cq values of replicates should vary by no more than 0.2 standard deviation units (Cq values)
- Identify the optimal annealing temperature by testing the amplification efficiency and reproducibility across a range of temperatures
- Verify assay specificity by running a melt curve



Temperature	Efficiency	$R^2$	Slope	Y-intercept
60.0°C	98.6%	1.000	3.357	34.294
60.7°C	97.1%	1.000	3.392	34.705
62.0°C	97.6%	0.999	3.381	34.529
64.0°C	95.6%	0.999	3.431	34.825
66.4°C	85.7%	0.995	3.719	37.715
68.4°C	61.3%	0.996	4.816	50.900
69.5°C	33.8%	0.872	7.898	79.112

## 7 VALIDATING ASSAY EFFICIENCY USING AN INCORRECT RANGE OF STANDARD DILUTIONS



Unknown 2 is outside the linear range of the standard curve. Therefore, the PCR efficiency of the reaction is unknown for this sample.

A standard curve is used to determine the efficiency, linear range, and reproducibility of a qPCR assay. These values are valid only for the concentration range of the serial dilutions used to generate the standard curve. The reaction efficiency cannot be extrapolated to samples that have a Cq outside the Cq range of the serial dilutions.

### How to avoid

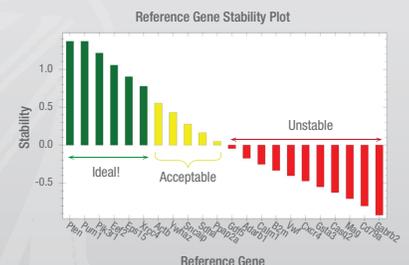
- Evaluate a range of standard dilutions that span the expected concentration range of your target
- Prepare sequential dilutions (5- to 10-fold) that span at least 5 orders of magnitude
- Pipet the same volume of DNA for each dilution
- Use the correct size pipet, especially for small volumes
- Avoid pipetting less than 5  $\mu$ L
- Use water in place of DNA for a negative control to detect contaminants

## 8 USING AN UNSTABLE REFERENCE GENE

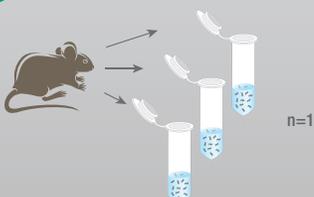
Not all commonly used reference genes are stable under all conditions. Failing to confirm the stability of a reference gene may produce biologically irrelevant results.

### How to avoid

- Validate the suitability of any reference gene to confirm its stability
- Run a preplated reference panel containing a set of commonly used reference genes to identify the most suitable reference genes for your experimental condition
- Use more than 1 reference gene that does not change expression as a result of the experimental treatment or condition



## 9 FORGETTING TO USE TECHNICAL REPLICATES



Confidence in the measurement and process is lacking without repeated measurement of each sample to evaluate the precision and reproducibility of an assay.

### How to avoid

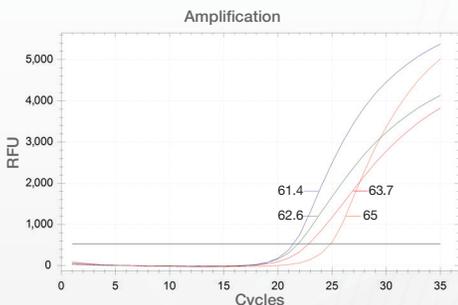
- Run each sample in triplicate (minimum)
- Perform a power analysis to determine how many replicates you need to see a certain fold change

## TIPS, TRICKS & BEST PRACTICES

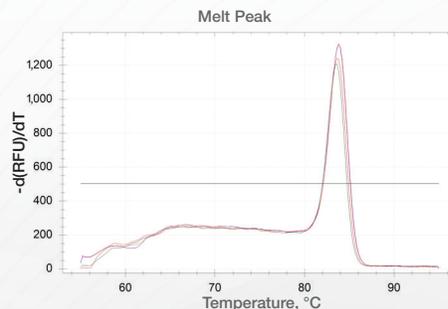
# 2-Plate qPCR Assay Optimization and Validation

### PLATE 1: OPTIMIZE PRIMER T<sub>a</sub> USING A THERMAL GRADIENT

- Annealing temperature (T<sub>a</sub>) must be high enough to ensure primer specificity, but low enough for efficient PCR
- Use thermal gradient feature to test a range of temperatures above and below target T<sub>a</sub> of primers (e.g., 55–65°C for primers with a target T<sub>a</sub> of 60°C)
- Run a melt curve to ensure specificity of primers using SYBR® Green
- Select T<sub>a</sub> that gives lowest C<sub>q</sub> value without generating nonspecific amplification (Figure 1)
- Inspect melt peak for target T<sub>a</sub> to verify presence of 1 tall, narrow peak (Figure 2)



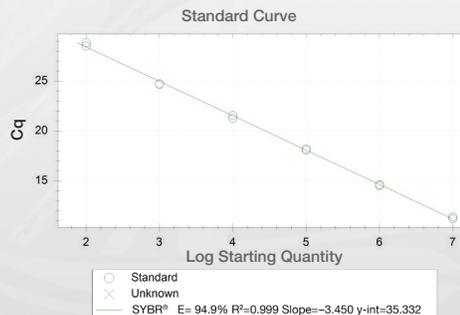
**Figure 1:** Assay optimization of primers using a positive control sample run along a thermal gradient. Here, 61.4°C is optimal because it is the warmest temperature that gives lowest C<sub>q</sub> (21 compared to 22 and 25 for higher temperatures).



**Figure 2:** A melt peak can verify specificity of a primer set. A specific assay will only amplify one PCR product and thus have one melt peak. Multiple peaks indicate multiple products such as off-target amplicons or primer-dimers. Off-target amplification may be corrected by running PCR at a higher T<sub>a</sub> or may require redesign of primers.

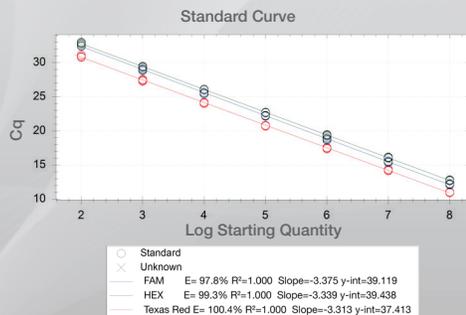
### PLATE 2: VERIFY ASSAY EFFICIENCY BY RUNNING A STANDARD CURVE

- Create a serial 10-fold dilution of a positive control sample
- Run qPCR on all dilutions of sample using optimized T<sub>a</sub> for primers
- Plot C<sub>q</sub> values against the log of starting concentration and generate a best fit line (Figure 3)
- PCR efficiency (E) can be calculated  $E = 10^{(-1/\text{slope})}$
- % Efficiency = (E - 1) × 100%. Assay efficiency should range from 90 to 110%
- Coefficient of determination (R<sup>2</sup>) for standard curve should be R<sup>2</sup> > 0.98



**Figure 3:** A standard curve can be generated with a serial dilution of template DNA. An inverse correlation exists between the log of the quantity of DNA (x-axis) and the C<sub>q</sub> value (y-axis). PCR efficiency can be calculated from the slope of the standard curve and utilized for calculating relative quantity (Pfaffl Method).

### PROBE MULTIPLEXING: SPECIAL CONSIDERATIONS



**Figure 4:** qPCR multiplex assays must be validated and optimized together to verify that PCR assay efficiencies are not inhibited due to competition.

- Melt curves are only compatible with intercalating dyes (e.g., SYBR® Green) and cannot be performed with hydrolysis probes
- Multiplex assays will need to be validated together to ensure that competition between assays is avoided (Figure 4)

#### Suggested Reading

Bustin SA et al. (2009). The MIQE guidelines: Minimum information for publication of quantitative real-time PCR experiments. *Clin Chem* 55, 611–622.

Taylor SC et al. (2019). The ultimate qPCR experiment: Producing publication quality, reproducible data the first time. *Trends Biotechnol* [published ahead of print Jan 14, 2019]. Accessed April 23, 2019.

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