applied biosystems

WHITE PAPER

Design and optimization of SYBR Green assays

For qPCR measurement of relative gene expression displayed across two lines

This guide is intended to help with the design and optimization of scientifically sound qPCR assays with SYBR™ Green detection. By following the steps in this guide, you may have a higher level of confidence that experimental results are based on concentrations of target sequences, and not on limitations or biases introduced by enzymes, reagents, and most notably, assay design.

Reverse transcription: beware of RT bias

Nearly all reverse transcription (RT) enzymes have the potential to introduce RT bias. If this happens, the amount of cDNA will not be in alignment with the amount of RNA in samples. When using relative quantitation methods, it is especially important to make sure that conclusions are based on your experimental treatments and not on limitations or bias of the RT enzymes.

How to test for RT bias

1. Step 1: Reverse-transcribe 2-fold dilutions of a known amount of RNA
2. Step 2: Run a qPCR standard curve (Figure 1) for each assay and an endogenous control

62.5 ng RNA → cDNA

125 ng RNA → cDNA

250 ng RNA → cDNA

500 ng RNA → cDNA

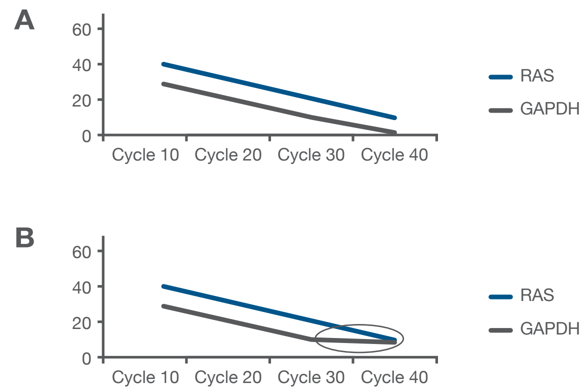


Figure 1. Experimental determination of RT bias. Reverse transcription reactions were run on dilutions of the indicated RNA samples. The resulting cDNA was used for qPCR standard curves. **(A)** The two qPCR standard curves are parallel for all concentrations, indicating no RT bias. **(B)** An example of RT bias. This simple test will reveal RT bias and is an important test that should be done for each experimental assay. This test also advises how much RNA you can use and still maintain consistent RNA:cDNA ratios for qPCR. If the purification scheme changes, the test should be repeated.

Shortcut

Invitrogen™ SuperScript™ VILO™ Master Mix (Cat. No. 11755050) enables linear amplification from 0.1 pg to 2.5 µg, the largest dynamic range in the industry. There is no need to test for RT bias with SuperScript VILO Master Mix.

ThermoFisher SCIENTIFIC

SYBR assays, step 1: bioinformatics

1. Using a sequence masking tool such as SNPmasker (**bioinfo.ebc.ee/snpmasker/**), pull up the sequence for the gene of interest, and select an exon-to-exon spanning region ~200 bp in length.
2. Mask for single nucleotide polymorphisms (SNPs). SNPmasker will highlight any SNPs that occur.
3. To avoid specificity issues, utilize a tool like RepeatMasker (**repeatmasker.org**) that will look for runs of Cs and Gs.

RAS

```
atacaaggatgcgtacagtacat
tcagacgaatggccgatagagc
gcatatcgcgaacatcgcgcata
tcgcgctaaagcgcctaagcgg
gcctaaaaggctcttccgcaaac
atatacgcgcagtgcgcgcttac
gaaggattggccattaggattag
cccgccaggggattgagagcc
agcccagcttagctcgatcgaac
gactacaggctacatatataacg
ccgaattagccaggattatgcca
ggggtaattcagacaacaaa
```

4. Now, take this qualified sequence and insert it into a primer designer such as Primer3web (**bioinfo.ut.ee/primer3/**). This should give you multiple sets of forward and reverse primers. Pick several for step 5.
5. Use the BLAST™ tool for primers (**blast.ncbi.nlm.nih.gov/**) to ensure that the primers chosen in step 4 are specific for your gene and unique to your species of interest.
6. Order your primers from **thermofisher.com/oligos**

Estimated total bioinformatics time: 1–2 hours
Estimated reagent usage: 0

Shortcut

See page 3 for information on how to eliminate all bioinformatics steps.

SYBR assays, step 2: primer validation

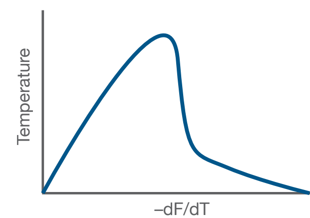
In primer validation, the objective is to find the right concentration of forward and reverse primers that will yield the lowest C_t and create no primer-dimers.

1. Run multiple qPCR reactions with 3 to 4 different concentration contributions of forward and reverse primers. Actual concentrations may vary from the example below. The appropriate range of primer concentration is determined by the master mix.

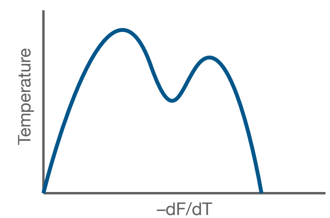
Forward primer

Reverse primer	300 nM	500 nM	800 nM
300 nM			
500 nM			
800 nM			

2. Evaluate C_t for each combination.
3. Run a melting curve for each combination.



Good result: Single peak indicative of a single PCR product.



Unfavorable result: Multiple peaks indicative of more than one PCR product being produced. SYBR Green dye will intercalate into both products and produce a signal.

If melting curve analysis shows primer-dimers, there are two options:

A. Start over with the bioinformatics.

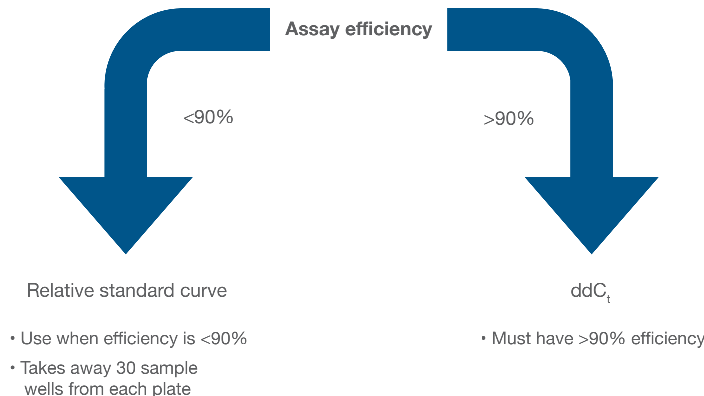
B. Alter cycling temperatures to remove primer-dimers. However, this may result in assays that run with different cycling temperatures and so cannot be combined with other qPCR assays.

Shortcut

See page 3 for information on how to eliminate all primer validation steps.

SYBR assays, step 3: assay efficiency

There are two primary methods of relative quantitation: relative standard curve and $\Delta\Delta C_t$ (ddC_t). The efficiency of the assay (ability to double the amount of DNA/cDNA in every cycle) will determine which method can be used.



Important

The final calculation in $\Delta\Delta C_t$ is $2^{-\Delta\Delta C_t}$. The "2" implies a perfect doubling of DNA/cDNA in each cycle of the assay. If assay efficiency is <90%, it is not actually doubling every cycle and the equations for $\Delta\Delta C_t$ are no longer valid, requiring either a new start (with bioinformatics) or use of a relative standard curve. It is critical to determine assay efficiency if using $\Delta\Delta C_t$.

How to validate assay efficiency

1. If necessary, reverse-transcribe RNA to cDNA.
2. Run a 5-point standard curve, in triplicate, using 10-fold dilutions for both the target gene and a reference gene.
3. Plot C_t values versus concentration to generate a standard curve for both target and reference gene (using qPCR software). Look for efficiency of >90% (to use $\Delta\Delta C_t$). Slope values for the target gene and the reference gene should be within 0.1 of each other.

Shortcut to all design and optimization steps with TaqMan Assays

Bioinformatics shortcut: Applied Biosystems™ TaqMan™ Assays minimize the need for more bioinformatics, because each assay has undergone our extensive 7-layer bioinformatics process before it arrives at your bench.

Primer validation shortcut: TaqMan Assays help avoid primer validation, because the combination of primers and probe is so highly specific that your qPCR instrument should detect only your target of interest.

Assay efficiency shortcut: TaqMan Assays are guaranteed to offer efficiencies of >90%.

TaqMan Assays are affordable

For less than you think, you can order a 75-reaction TaqMan Assay and start running experiments immediately upon arrival. When you consider all the time, reagents, and samples (39 qPCR reactions: ≥9 for primer validation, 30 for assay efficiency) required to optimize a single SYBR Green assay, TaqMan Assays offer tremendous value. This is especially true if you find it necessary to start over at any point of the SYBR Green assay design and validation process.

TaqMan Assay guarantees:

- Sensitivity: 10 copies
- Assay efficiency: >90%
- Dynamic range: 7 logs (we have demonstrated 9)
- Ease of use: all TaqMan assays have the same cycling protocols

PowerUp SYBR Green Master Mix

Applied Biosystems™ PowerUp™ SYBR™ Green Master Mix is formulated for maximum specificity and reproducibility:

- Exceptional specificity with a dual hot-start mechanism
- Tight reproducibility in C_t values over a broad dynamic range
- Compatible with standard or fast cycling for results in less than 50 minutes
- Formulated with UNG/dUTP to prevent contamination of carryover PCR products
- Stable at room temperature for 72 hours after plates are prepared for qPCR
- Broad instrument compatibility

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Ordering information

Product	Quantity	Cat. No.
PowerUp SYBR Green Master Mix, 2-Pack (2 x 1 mL)	200 reactions	A25779
PowerUp SYBR Green Master Mix, 1-Pack (1 x 5 mL)	500 reactions	A25742
PowerUp SYBR Green Master Mix, 5-Pack (5 x 1 mL)	500 reactions	A25780
PowerUp SYBR Green Master Mix, 2-Pack (2 x 5 mL)	1,000 reactions	A25776
PowerUp SYBR Green Master Mix, 10-Pack (10 x 1 mL)	1,000 reactions	A25918
PowerUp SYBR Green Master Mix, 5-Pack (5 x 5 mL)	2,500 reactions	A25777
PowerUp SYBR Green Master Mix, Bulk Pack (1 x 50 mL)	5,000 reactions	A25743
PowerUp SYBR Green Master Mix, 10-Pack (10 x 5 mL)	5,000 reactions	A25778

For more information about PowerUp SYBR Green Master Mix and additional SYBR Green master mix formulations, visit **thermofisher.com/sybr**

To learn more about TaqMan Assays, visit **thermofisher.com/taqman**

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