Design and optimization of SYBR Green assays

For qPCR measurement of relative gene expression

This guide is intended to help researchers design and optimize scientifically sound qPCR experiments with Applied Biosystems™ SYBR™ Green assays. These recommendations are consistent with the Minimum Information for Quantitative Experiment (MIQE) guidelines. By following the steps in this guide, you may be more confident that experimental results are based on the concentrations of target sequences, without limitations or biases introduced by enzymes, reagents, and, most notably, assay design.

Reverse transcription (RT)—beware of RT bias

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

How to test for RT bias

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

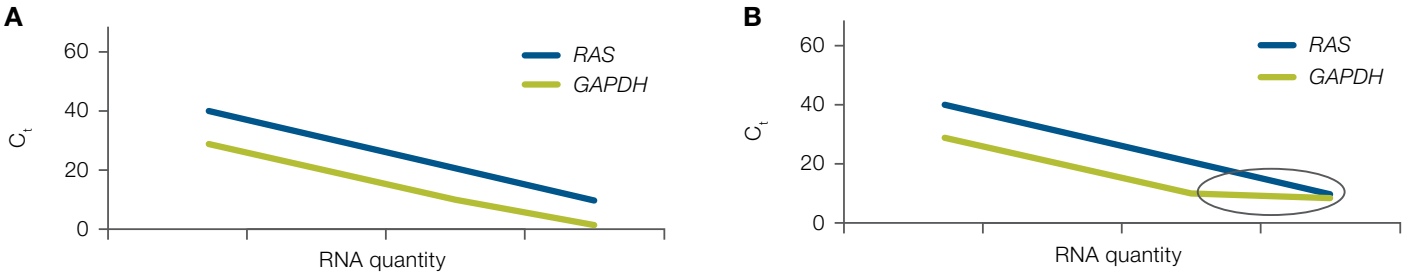


Figure 1. Experimental determination of RT bias. RT reactions were run on a dilution series of RNA. The resulting cDNA was used to construct qPCR standard curves. **(A)** The two qPCR standard curves are parallel for all concentrations, indicating no RT bias. **(B)** An example of RT bias is indicated by the oval. Constructing this RT-qPCR standard curve will reveal if you are introducing RT bias, and is an important check that should be done for each experimental assay. The standard curve will also indicate how much RNA you can use and maintain linearity for qPCR. If the purification scheme changes, the standard curve check should be repeated.

SYBR Green assays, step 1: bioinformatics

1. Obtain the sequence for the gene of interest, and select an exon-to-exon spanning region ~200 bp in length.
2. Use a tool such as SNPmasker (**bioinfo.ebc.ee/snpmasker/**) to mask for single-nucleotide polymorphisms (SNPs). SNPmasker will highlight any SNPs that occur.

Example sequence for *RAS*

atacaaggatgcgtacagtacattcagacgaatggccgatagagc
gcatatcgcgaacatcgcgcatatcgcgctaaagcgcctaagcg
ggcctaaaaggctcttccgcaaacatatacgcgcagtgcgcgcttac
gaaggattggccattaggattagcccgccaggggattgagagc
cagcccagcttagctcgatcgaacgactacaggctacatatataac
gccgaattagccaggattatgccaggggtaattcagacaacaaa

3. To avoid specificity issues, utilize a tool like RepeatMasker (**repeatmasker.org**) that will look for runs of Cs and Gs.
4. Now take this qualified sequence and insert it into a primer design tool 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.

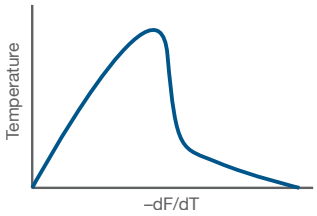
SYBR Green assays, step 2: primer validation

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.

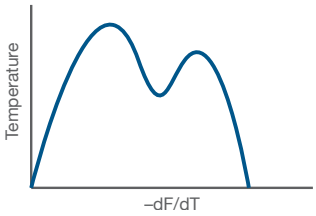
1. Run multiple qPCR reactions with 3 to 4 different concentrations of forward and reverse primers. Actual quantities may vary from the example below. The appropriate range of primer concentrations is determined by the master mix.

Reverse primer \ Forward primer	300 nM	500 nM	800 nM
100 nM	Reaction 1	Reaction 2	Reaction 3
250 nM	Reaction 4	Reaction 5	Reaction 6
500 nM	Reaction 7	Reaction 8	Reaction 9

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



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



Unfavorable result: multiple peaks indicative of more than one PCR product. SYBR Green dye will bind to both products and produce a signal.

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

– Start over with the bioinformatics.

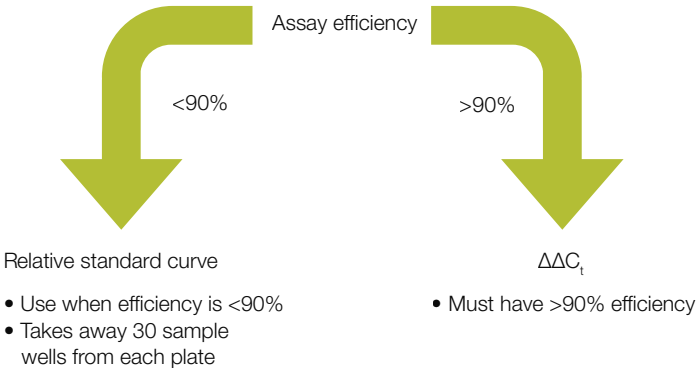
– Alter cycling temperatures to remove primer-dimers. However, this may result in assays that run with different cycling temperatures and so cannot be combined with other qPCR assays.

Shortcut

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

SYBR Green assays, step 3: assay efficiency

There are two primary methods of relative quantitation: relative standard curve, and comparative C_t ($\Delta\Delta C_t$ or ddC_t). The efficiency of the assay (ability to double the amount of PCR product 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 perfect doubling of DNA with each cycle of the assay. If assay efficiency is <90%, it is not doubling with 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 vs. concentration to generate a standard curve for both target and reference genes (using qPCR software). Look for >90% efficiency (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 the need for 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. When you consider all the time, reagents, and samples** 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 logarithmic units (we have demonstrated 9)
- **Ease of use:** all TaqMan Assays have the same cycling protocols

PowerTrack SYBR Green Master Mix

Applied Biosystems™ PowerTrack SYBR™ Green Master Mix is formulated for maximum real-time PCR specificity and reproducibility, and includes a tracking dye system to minimize pipetting errors.

- Built-in two-color tracking dye system to help improve pipetting accuracy and reaction setup
- Broad primer T_m and primer concentration compatibility allows qPCR reaction setup flexibility with minimal optimization
- Superior specificity and tight reproducibility in C_t values over a broad dynamic range improve data quality
- Compatible with Invitrogen™ SuperScript™ IV VILO™ Master Mix reverse transcription for fast, reproducible results
- Formulated with UNG and dUTP to prevent contamination of the reaction by carryover PCR products
- Broad instrument compatibility

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

* For exclusions, please see the TaqMan Guarantee Terms and Conditions at **thermofisher.com/us/en/home/brands/taqman/taqman-guarantee/taqman-guarantee-terms-conditions.html**

** 39 qPCR reactions (9 or more for primer validation and 30 for assay efficiency)

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Ordering information

Product	Quantity	Cat. No.
PowerTrack SYBR Green Master Mix, Mini Pack (1 mL)	100 reactions	A46012
PowerTrack SYBR Green Master Mix, 1-Pack (1 x 5 mL)	500 reactions	A46109
PowerTrack SYBR Green Master Mix, 2-Pack (2 x 5 mL)	1,000 reactions	A46110
PowerTrack SYBR Green Master Mix, 5-Pack (5 x 5 mL)	2,500 reactions	A46111
PowerTrack SYBR Green Master Mix, 10-Pack (10 x 5 mL)	5,000 reactions	A46112
PowerTrack SYBR Green Master Mix, Bulk Pack (1 x 50 mL)	5,000 reactions	A46113

Find out more about TaqMan Assays
at **thermofisher.com/taqman**

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