Troubleshooting Forum

Molecular Biology Techniques Q&A

Real-Time qPCR/qRT-PCR Methods: Standard Curves

This month's question from the Molecular Biology Forums (online at molecularbiology.forums.biotechniques.com) comes from the "Real-Time qPCR/qRT-PCR Methods" section. Entries have been edited for concision and clarity. Mentions of specific products and manufacturers have been retained from the original posts, but do not represent endorsements by, or the opinions of, *BioTechniques*.

Is it necessary to run standard curves for qRT-PCR? (Thread 22909)

Q I am using SYBR Green and the $\Delta\Delta C_t$ method to detect expression changes in 10 genes. It seems nearly impossible to get my primer efficiencies approximate between my 10 genes and endogenous controls. To draw a standard curve for each gene would require 308 SYBR Green reactions and at least one week. I have considered using Taqman since I heard that I would not need standard curves for relative quantification. Is this true?

None of my primers show dimers in melting curves or on agarose gels. C_t values vary from 19 to 31 and are always consistent.

Should I switch to Taqman or continue with SYBR?

A Since you have already demonstrated good primer design, it is best to continue with SYBR.

All qPCR approaches should definitely use standard curves. Run the standard curves once for all targets and then run your unknowns. Be certain that your unknowns are all diluted to the same concentration that falls within the standard curve.

Q ABI says it guarantees $100 \pm 10\%$ efficiency for all of its pre-made Taqman assays. ABI's SDS program (2.0) runs an algorithm to account for the differences in efficiency in each assay. I can either run Taqman and trust that all assays will be nearly perfect, or run SYBR and trust that the software will calculate everything. Either way I don't need standard curves. It is true that all my primers work well, but isn't Taqman a more robust assay that will give me more accurate results?

A Just because you run a TaqMan assay doesn't guarantee that it will be 100% efficient. You must first show that there is no inhibition and demonstrate perfect fidelity of the probe and accompanying primers for their respective targets by BLAST.

No qPCR is valid or quantitatively informative when inhibition is present. If there is inhibition, the most concentrated sample points in your standard curves will appear higher than expected and the line will curve upward. You can use the Nolan-Bustin "SPUD" assay to test for inhibition.

Depending on the master mix you use, the primers will behave differently. Just because ABI guarantees high efficiency with each of their validated primer sets doesn't mean that a master mix from another manufacturer will be able to perform as well. I design all my primers and probes using ABI Primer Express v. 2.0. They work perfectly with Invitrogen and Quanta BioSciences master mixes, but behave poorly with the ABI One-Step kit with the MultiScribe RT enzyme.

I personally run standard curves every single time for every single target. Given that samples age as they sit, I think that it is good to see how the standards behave as time progresses.

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Also, I never rely on machine software to calculate my final results. I insist on staying directly familiar with the math involved in case the machine interprets something incorrectly. I get the same results as the machine with my own Excel files when everything is working perfectly and when inhibition is absent.

Q I ran assays with serial dilutions to check the concentration of cDNA I needed. The curves shifted accordingly until I reached the limit and from the results, I drew standard curves in Excel that gave me straight lines. If my C_q values are always consistent, does this indicate the absence of inhibition?

Do you have your Taqman plates custom-made with extra wells for your standard curves?

A Yes, that is an appropriate test for inhibition.

I create my own Taqman plates. I set up everything in duplicate. I order the primer lyophilates and Taqman probes from ABI and use each primer at 775 nM and the probes at 150 nM. The pre-formulated ABI primer-probe mixes have 900 nM primers and 250 nM probe in the final reactions.

How do you make your cDNAs? I used a MultiScribe/AmpliTaq Gold Taq-based master mix for 5 years until I realized that my sensitivity could be increased up to a million-fold by switching to an SSIII- or QuantaScript-containing one-step master mix, which actually cost less.

Q I ran SYBR assays for standard curves of all 10 genes in triplicate with seven 1:2 serial dilutions. My first plate showed efficiencies ranging from 92% to 108%. I made more cDNA from RNA frozen at -80°C and ran the plate again with the new cDNA. I used exactly the same amounts, conditions, etc., but efficiencies decreased dramatically. C_t values also varied dramatically.

What is the relation between a change in C_t value and efficiency? Gallup and Ackermann (Gallup, J.M. and M.R. Ackermann. 2006. Addressing fluorogenic real-time qPCR inhibition using the novel custom Excel file system 'FocusField2-6GallupqPCRSetupTool-001' to attain consistently high fidelity qPCR reactions. *Biol Proceed.* Online 8:87-155) observed a decrease in C_t values using frozen-thawed RNA for their reactions, but they do not mention changes in efficiencies. Have you ever observed a decrease in C_t and efficiency?

I also noticed a loss in log linearity of my standard curves for some genes when I used the highest or lowest dilution points to graph the data. Gallup et al. (Gallup, J.M., F.B. Sow, A. Van Geelen, and M.R. Ackermann. 2009. SPUD qPCR assay confirms PREXCEL-Q software's ability to avoid qPCR inhibition. *Curr. Issues Mol. Biol.* 12:129-134) and other publications talk about inhibition of qPCR due to high or low cDNA concentrations. They argue that there is a best fit for each target within a standard curve and that the PREXCEL-Q software will calculate this. Do you use this software to define your standard curves?

A I use PREXCEL-Q for all of my qPCR experiments because I invented it. I am Jack M. Gallup. If you would like a free copy of the program, I can send it to you.

I don't freeze RNAs at all for the very reasons you've cited. I have also seen the changes in efficiency with age or freeze-thawing of samples.

Standard curves become less efficient at high dilutions because PCR becomes more stochastic in the presence of less template.

Selected and edited by Kristie Nybo, Ph.D.

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