Troubleshooting Forum

Molecular Biology Techniques Q&A

Real-Time qPCR/qRT-PCR Methods: Amplification in Template-Free Controls

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 they do not represent endorsements by, or the opinions of, *BioTechniques*.

How can I eliminate expression in my no-template controls? (Thread 21619)

Q I have several problems with my qPCR, including amplification in the no-template control reactions (these wells show peaks in the melting curve analysis) and unacceptable technical replicate differences in the Ct values.

I tried total RNA extraction and Dynabeads from Invitrogen and prefer the latter since it returns better quality RNA. I check my material with the NanoDrop and Bioanalyzer, retrotranscribe using SSII and oligo dT20, and use the MX3000P system from Stratagene with SYBR Green II.

My material has no genomic contamination, verified by running PCR with suitable primers, so I think that the variability might be caused by poor cDNA. Does anyone have any suggestions?

A Did you run the reactions on a gel? The amplification you are seeing may be primer dimers, which will have a melting peak. You might be using too much primer.

The variation in your replicates might be caused by pipetting error. Are you trying to pipette very small volumes (below 1 μ L) of template? Try using a pipettor meant for 1–10 μ L volumes.

If you think the issue is the cDNA, try using a mix of random primers and oligo dT. Qiagen supplies this mix with the QuantiTect reverse transcription enzyme and BioRad does also with iScript.

Q I have tried several primer dilutions to try to avoid primer dimers and nonspecific amplification: 150 nM, 250 nM, and 500 nM. At 150 nM, the whole plate fails.

I tried 1 and 2 μ L of cDNA with new pipettors. I always make a master mix and add the cDNA at the end. Adding 2.5% DMSO helps, but only a little. Using more DMSO inhibits the reaction.

A If you are making a master mix, the replicates should not differ. ROX dye should normalize for differences in pipetting the master mix. Try triplicates instead of duplicates and make sure everything is well mixed before pipetting. Try the Qiagen SYBR green master mix. I got almost identical replicates with it.

You might have problems with wells at the edge of the plate if the tape is not sealed well or if the block is not heating well on the outside. Make sure the instrument is calibrated and running properly.

You can sometimes eliminate extra fluorescence by adding an extra data acquisition step at 77°C for about 5 seconds.

Q ROX dye helped correct the signal, but the results weren't affected much by its presence.

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I tried a different machine, running triplicates, and running the same genes in different positions on the plate and still get variable results. I don't think the problems are caused by irregularity in heating the plate. I also tried very short annealing and extension steps of about 5–10 seconds.

A I wouldn't try shorter annealing or extension steps unless it is recommended for your instrument. What does your no-RT control look like?

Plant RNA may have more secondary structure than other RNAs. I have heard good feedback on the Invitrogen Thermoscript RT, which can be used at higher temperatures to help remove secondary structure. Qiagen RT reads through secondary structure at lower temperatures.

Q I tried Thermoscript plus oligo dT20, SuperscriptII plus hexamer and oligo dT20, and the Quantitect reverse transcriptase kit from Qiagen with oligo plus nonamers. In general, I did not see a big difference between enzymes and I still have the same problems.

I added an extra 81°C step after elongation and acquired fluorescence at the end of that step. This got rid of most (but not all) of my no-template control amplification.

A1 Phenolics and polysaccharides in your original plant extracts can carry over into your RNA samples and impede your RT reactions.

To prevent this, dilute all of your RNA samples 1:30 after RNA extraction and DNase treatment. From there, dilute all of the samples to the same concentration regardless of what is suggested for RT. Use the exact same concentration of RNA for each RT reaction. All of your RT reactions will then behave similarly and your cDNAs will not cause problems in your final qPCR reactions. You can dilute all of the resulting cDNAs 1:100 before qPCR.

A2 Try dUTP. It is included in some mixes like Qiagen and ABI. The PCR product will have U incorporated in the strand. Then you can perform a 2-minute digest with uracil-N-glycosylase before activating the *Taq* and any contaminating PCR product will be digested away.

A3 You might want to explore the possibility that you have splice variants of your targets of interest.

A4 Look at the papers by Bustin (2002; *J. Mol. Endocrinol.* 29:23-39) and Wong and Medrano (2005; *BioTechniques* 39:75-85). These show that the biggest source of variation in qPCR is the researcher performing the experiment. If you have signal in the no template control and the replicates differ, then you are probably just missing something very simple like vortexing the SYBR green mix or master mix. The quick vortex spin steps make a lot of difference.

A5 Your plates and tubes are very prone to cross-contamination. Try leaving empty wells around your no-template controls.

A6 My secret is the white-welled plates from Eppendorf. They give higher signal, tighter replicate Cts, and are cheaper than ABI's plates by almost 50%. Cts are also slightly lower because of higher reflectivity of the signal from the white welled surface. The only drawback is that it is harder to see into which wells you have already dispensed your reagents.

A7 Try re-autoclaving your water or TE diluent solution that you use in your qPCR. Reagent contamination is a major cause of no-template control signal and the offending reagent is usually the diluent water or TE.

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