Troubleshooting Forum

Molecular Biology Techniques Q&A

qPCR: Single copy targets

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*.

How can I increase the sensitivity of my TaqMan assay enough to detect a rare template? (Thread 21795)

Q I have a mixture of DNA and need to amplify a particular sequence by qPCR. The target DNA makes up only 0.1% to 10% of the mixture. I have a set of primers that work well in terms of sensitivity and specificity for endpoint PCR. But when I use these same primers for SYBR green PCR, I see non-specific fluorescence and primer dimers in the gel.

To avoid these problems, I designed a TaqMan assay and I am currently trying to standardize it using a pure extraction of my target DNA at concentrations between 0.1 ng/μl and 20 ng/μl. The assay has several problems: there is no sensitivity in the lower range of concentrations, with no amplification at all when concentrations of the target are below 5 ng/μl; duplicates and triplicates present varied results, especially in samples with lower concentrations; and even when using concentrations of 20 ng/μl, the Ct values are above 30. I would like to decrease the Ct values to 26 or lower and improve the levels of detection.

I tried using Invitrogen SYBR green qPCR supermix-UDG, Abgene's Absolute Fast qPCR Low ROX Mix, and Absolute qPCR Low ROX Mix.

The problem in the replicates is not caused by pipetting errors since I work from master mixes. I thought if the DNA was insufficiently dissolved and suspended, that might be the cause of the problems. So I tried vortexing and spinning each diluted sample and then left them overnight at room temperature. I used the NanoDrop to quantify the samples twice, once immediately after vortex-centrifuging and a second time after overnight incubation at room temperature. The measurements were taken in duplicates. The NanoDrop's range of detection is 2-3700 ng/μl for dsDNA in a single sample, so I can't quantify <2 ng/μl. Also, the results of quantification before and after overnight incubation are not reproducible.

How can I improve this assay so that the replicates will show identical results, concentrations as low as 0.1 ng/μl DNA will be detected, and Ct values will appear around 26?

A Try designing two or three more primer sets to see if another pair will work better. You can spend a lot of time optimizing for one primer set, but for \$10, you might order another set that works perfectly the first time. Even if you optimized for conventional PCR, the conditions are different for qPCR, and you may need

to use different temperatures. Since you have tried three different systems and annealing temperatures, it sounds like that primer set should be redesigned. Another possibility is a problem with the template. Did you run a positive control?

Have you been able to measure the PCR efficiency? Try taking a PCR product that shows a clean single band, column purify it, make serial dilutions, and use this for a standard curve if you have no other good control. Then you will be able to determine the efficiency of the primers. The irreproducible OD readings on your samples were probably caused by insufficiently dissolved DNA in the initial reading. There is no need to quantify the undissolved DNA. You might also try a few methods for cleaning the DNA if you think you have contaminants. Pure DNA is readily soluble in water; even large genomic DNA should still dissolve with overnight incubation or heat.

Q I have four primers (two forward and two reverse) and have tried combinations of these. The problems I described are seen with the best working pair. I need to design the primers in a very limited area of the sequence in order to avoid binding to several closely related sequences. The main requirements for the primers are selectivity and specificity in the presence of several closely related sequences in the template. Because of these restrictions, I wasn't able to design more than these two pairs.

The PCR efficiency is low at lower template concentrations, but ranges from 90-99% at higher concentrations (>5 ng/μl).

I isolated genomic DNA using Qiagen spin columns. The DNA purity might be a problem since the 260:280 ratio was less than 1.7. What is the best way to clean this DNA?

A I also use the Qiagen kit to extract genomic DNA and it works well. After eluting with AE buffer, I put the samples directly onto a gel and usually see well-defined bands. In qPCR, the DNA amplifies with Cts between 23 and 26. I do not think your problem is related to DNA extraction; I think it is how the DNA is performing during PCR. Did you run a 0.8% agarose gel on your extracts? How was the band integrity?

In your master mix, be sure to use 5× the concentration of primer and probe. Also, be sure to use 95°C for strand separation, 65°C for annealing, and 72°C for extension. Run 45 cycles instead of 30. This should improve the sensitivity and lower the Cts.

A I agree that Qiagen kits work well, but depending on the sample source, the 230:260:280 ratios may not be optimal, so you will need to do further clean-up. Try ethanol precipitation or bind the DNA to a second column, then wash and elute the sample again.

Your primers only need to span the region where the TaqMan

probe will bind. You can also use a secondary product that does not bind the probe.

For measuring low DNA concentrations, you need to use a fluorometer and pico green.

A If your targets are rare, especially approaching single copy entities in your samples, the lowest Ct you can expect is 33.219, assuming that the level of sensitivity of the qPCR machine is 10 billion copies at threshold (as it is with most qPCR machines). After 33.219 cycles, one copy of the template will become 10 billion copies, meeting the threshold if the reaction performs at 100% efficiency. But, as you have noted, the less template there is, the lower the efficiency is, resulting in later Ct values. So, when working with such low concentrations, you will not see a Ct of 26, because it is currently impossible for any machine to detect one copy above background fluorescence before 33.219 cycles.

Are you using minor groove binding (MGB) non-fluorescent quencher probes? These usually come with 6FAM on the 5'-end and allow for the best and shortest probe design. Locked nucleic acid (LNA) MGB probes may be the best for your particular situation.

I recommend that you read *Journal of Microbiological Methods* 66 (2006) 206–216. These authors conclude that LNA-MGB TaqMan probes are the most effective for rare templates. The 6FAM-LNA-MBG probe will give you the lowest signal-to-noise ratio and the best Tms for discriminating between very similar sequences, which means you will have the best chance of seeing the single copy Ct at 33.219 cycles.

In cases where only 0.1% of your sample is expected to contain your target, you should dedicate an entire qPCR plate to that sample. That way you can use 100% of your sample and significantly increase your chance of finding the rare target in one of the wells. Even when you have the reaction working perfectly, you should still expect variable Cts in your rare copy samples.

In my opinion, your best chance at success would be to use a Takara, Invitrogen, or Quanta BioSciences qPCR Master Mix with a 6FAM-LNA-MBG probe (I believe you can order these from Exiqon), and the accompanying best primer pair to discriminate your target sequence of interest.

A Lockey et al.'s "Real-time fluorescence detection of a single DNA molecule," published in *BioTechniques* (1998; 24:744-746), is a good paper to help in designing studies to detect single copy templates.

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