Troubleshooting Forum

Molecular Biology Techniques Q&A

qRT-PCR with saliva samples

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*.

What is an appropriate reference gene to use for determining gene expression levels in saliva by real-time PCR? (Thread 31980)

Q I successfully detected expression of my gene of interest in peripheral blood mononuclear cells (PBMC) with qPCR, and am now trying to repeat the experiments with saliva using the same amplification protocols. I used SuperScript VILO for cDNA synthesis and Taqman for real-time PCR. The expression of all genes, including the 18S housekeeping gene, was significantly blunted compared to PBMC. I saw Ct values >37 for our gene of interest, and nearly 18 for 18S expression in saliva samples. In PBMC, the Ct was around 10. The standard curves for 18S look reasonable, however the sensitivity is really poor.

I tested the Taqman real-time step for inhibitors by spiking the saliva cDNA with cDNA from another source and found that the Ct values correlated with the amount of added cDNA. I also measured RNA integrity using Nanodrop and denaturing agarose gel electrophoreses, and it looked fine. I think the problem lies in the cDNA synthesis.

Across different human cell types, should 18S expression be similar? Is there a way to check for successful reverse transcription?

A Yes, 18S expression should be the same across all types of human cells.

You can test the success of first strand cDNA synthesis by adding a trace of radioactive deoxynucleotide or simply amplifying a gene using primers that span an intron.

cDNA synthesis can fail because of hairpin formation in the RNA. In this case, try to use Thermoscript works at higher temperatures; you might try using it instead.

Degradation of the RNA during reverse transcription could also cause this problem; try adding RNase inhibitor to your RT reaction.

A Last year, I worked on a project using RNA extracted from pig saliva. That RNA inhibited qPCR, so our challenge was finding an appropriate one-step kit. Since saliva contains several PCR inhibitors, you need to sufficiently purify the RNA. If not, it can carry its inhibitory substances into your VILO RT reactions and inhibit the SuperScript III enzyme. Saliva also carries RNases, so you will need to counteract this early in the RNA isolation process.

You might also check the RNA quantity added to the RT reaction. Too much RNA in the VILO can shut down the reaction completely, and if the RNA also has reverse transcriptase inhibitors present, adding more RNA will add more inhibitors, leading to further problems.

Q I used RNeasy to extract RNA from the saliva, so I think it should not contain many contaminating factors. I previously used RNeaqueous kit and Trizol with no luck. Is there a PCR inhibitor that is invisible on gel or Nanodrop?

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I added 500ng of RNA to the 20 μ l VILO reaction. I did try lower amounts of RNA as well, but still experienced problems.

A We successfully extracted RNA from saliva for RT-PCR using the MagMAX pathogen kit.

A I recall from Dr. Stephen Bustin's 2009 book that HotTub DNA-dependent polymerase is less prone to blood-derived inhibition than others. You might try that with your saliva samples.

A Why are you using 18S for your control? You need more than one control gene. If you have not validated the control genes within your samples, you may not get the appropriate answers. I usually start with three controls and then use NormFinder or geNorm to pick the best one. The ddCts should be approximately 1 for the control; if not, the rest of the data is unreliable. The expression levels of the control genes and the gene of interest need to be fairly close in order to make an accurate comparison.

Did you use a website such as GeneCards to look at the expression profile of your gene? It may not be expressed significantly in saliva. The expression level of the control genes should be close to the level your gene is expressed.

Since you're using TaqMan, you can try a duplex assay with Vic-labeling and run your control and gene in the same tube.

You should also note that the Nanodrop won't give you any information on RNA integrity. The gel helps a little, but I prefer to send my samples to the core facility to run on the Bioanalyzer for the RIN score. Any sample with a score <7 will not work well for qRT-PCR.

The 8-cycle difference in your control gene may indicate degradation; try using Trizol along with an RNase inhibitor. Since you see amplification of the 18S cDNA, your cDNA synthesis is working. The main contaminants I think you need to worry about are EDTA and alcohol from the purification.

A You might get an 18S signal from mis-priming since the RNA is so abundant.

A I also analyze my RNA samples with the Agilent 2100 Bioanalyzer before using them for qRT-PCR. M.W. Pfaffl, et al. recently noted that a RIN of 5.5 and above suffices for qPCR. RINs >7 are needed for next-generation sequencing applications. But of course, the higher the number, the better the RNA will perform in any assay.

A Saliva, like other bodily fluids, has no cellular context. Therefore, the amount of target transcript is not connected to the amount of any other transcript as it would be in a cell. Because of this, the transcripts usually used in data normalization in cellular contexts are not valid.

I recommend you measure total RNA with a PicoGreen kit and normalize expression compared to total RNA. You might also express your data as a ratio of one target compared to another. Ratiometric assays can be very powerful when the correct conditions are met.

When dealing with inhibition issues, fight the impulse to add more sample to compensate for poor results. Instead, add less in 2-fold reductions and I believe you will see an improvement.

A I like the idea of using target ratios as suggested above. I have used that approach in the past and it worked well in a few studies.

I prefer RiboGreen for RNA quantification over PicoGreen, which is primarily used for dsDNA quantification.

Selected and edited by Kristie Nybo, Ph.D.

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