

GENOME TECHNOLOGY

Real-Time PCR Tech Guide

**A TROUBLESHOOTING GUIDE:
EXPERTS GIVE THEIR ADVICE ON HOW
TO CONDUCT REAL-TIME PCR**



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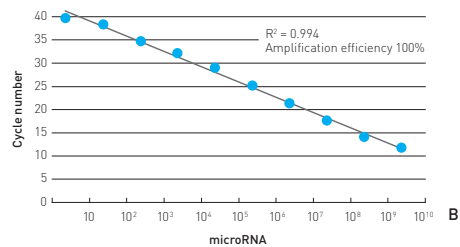
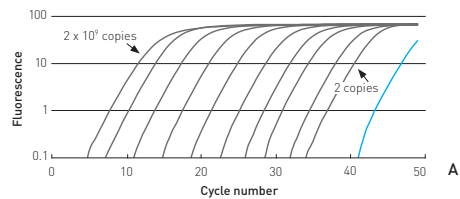
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Ina K. Dahlsveen, Ph.D., Product Manager



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Letter from the editor



For our sixth foray into PCR, we're going back to the basics. Over the past two or so years, we've covered all sorts of different aspects of PCR — from designing assays to using PCR in the clinic or using PCR for just microRNAs.

In this issue, we take a step back from the more niche aspects of PCR being trotted around out there. Instead, we're taking a look at the skills that hold up those more in-depth and specialized versions of PCR. As my teachers and

coaches used to say, you can only move on to more complicated endeavors after you've mastered the basics — you need to know how to do a layup before playing a game of basketball. Knowing the basic skills of PCR (good sample purity, validation, and normalization, among many others) keeps you on your game; they are the tenets of a well thought-out, properly designed experiment. Here, we have respected experts — first round draft picks, if you will — who offer their opinion on how to run a well-designed PCR experiment.

— Ciara Curtin

Index of experts

Genome Technology would like to thank the following contributors for taking the time to respond to the questions in this tech guide.



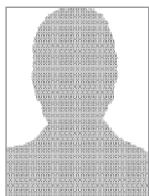
Mark Band

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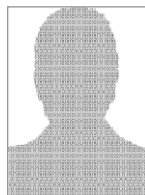
Christina Ferrell

MOLECULAR CORE FACILITY
SAN FRANCISCO VA MEDICAL CENTER



Ryan Sprissler

GENOMIC ANALYSIS AND
TECHNOLOGY CORE
UNIVERSITY OF ARIZONA



John Tine

MOLECULAR GENETICS
CORE FACILITY
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Thomas Wood

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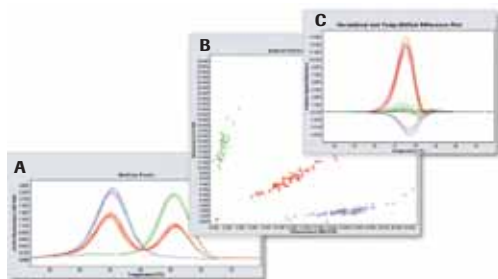


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Q1

How do you purify the DNA and/or RNA to be used?

Generally, most Trizol extraction protocols or commercial column kits are sufficient. Certain plant tissues may be more problematic and often PVP can be added to the Trizol to aid removal of metabolites. We generally recommend a DNase treatment. However, most extraction kits do an adequate job of DNA removal. We recommend a Bioanalyzer or equivalent analysis to determine RNA quality before any RT-PCR or array expression work.

— MARK BAND

DNA purification is a difficult one. I did some experiments to quantify transgene copy number in mouse tail tip sample and I found that DNA quality was absolutely critical for accurate results. Phenol/chloroform extraction was no good. DNasey column-based protocol

was not satisfactory either. We had the ABI extraction machine (vacuum based) on demo for a week and I tried it for DNA and for RNA. It was very good for DNA qPCR but I was not so convinced with the RNA.

RNA purification: I have used the Stratagene extraction kit for years. It is column based, and has the advantage of having a DNase treatment step included. It has worked fine for routine and qPCR. I have found that people using Trizol could face serious problems in reproducibility — and not in a consistent way. For example, one colleague had problems with RNA samples from different parts of the mosquito body following extraction with Trizol. I suggested she run a serial dilution with her two RNA samples for the cDNA reaction and to use a set amount of cDNA

in her qPCR. With one of her samples the correlation was excellent; with the other it was awful. It was clearly due to the quality of her RNA and likely due to phenol traces. So I suggested she clean her sample with a column-based kit. People talk about “assay validation.” I always add a step of “template validation” when using new tissues or new RNA extraction technique. This means that I use a previously validated qPCR assay and a serial dilution of the RNA for reverse transcription, and not a serial dilution of the cDNA.

— LAURENCE BUGEON

I prefer to use the filter systems available through Qiagen (Qiashrepper and RNeasy kits) or Promega (SV Total RNA). I have used other techniques and found that some genes are more

susceptible to degradation following isolation. Acid phenol or Trizol isolation not only captures less total RNA than the filters, but it also leaves a contaminating protein in the eluant. I have confirmed this using the Agilent Bioanalyzer 2100.

— CHRISTINA FERRELL

We generally purify our DNA through size exclusion filtration plates (Millipore) and resuspend in molecular grade water, but have found we get the absolute best results from a simple ethanol precipitation. The RNA is almost always extracted using Qiagen's RNeasy spin columns and eluted in RNase free water instead of their EB buffer. This is then pure enough to go directly into the RT reaction.

— RYAN SPRISLER

We routinely make use of the spin column-based kits provided by Qiagen and Ambion for purification of total RNA. If we have abundant sample we will treat with DNase as a component of the purification. We often omit this step, though, when the amount of sample is limiting,

“I have found that people using Trizol could face serious problems in reproducibility — and not in a consistent way.”

— Laurence Bugeon

because we want to minimize the loss of RNA that inevitably occurs during DNase treatment. In those cases, we rely on rational assay design to ensure that we do not detect background signal contributed by genomic DNA.

— JOHN TIME

We recommend the use of Ambion (now Applied Biosystems) RNAqueous for isolation of RNA. This is a very user-friendly protocol and has consistently produced high-quality RNA. There are a number of commercial products available for DNA isolation; however, we use a protocol originally developed by Bill McClements in George Vande Woude's laboratory in the late '70s. The story behind the method is that

they needed to process a large number of samples and Vande Woude wanted something that was not complicated, even if it required cutting a few corners. Thus the "Round Method" was born. Cell lysis (2ml/T25) is performed using 10mM TrisHCl pH7.5, 10mM EDTA 0.6 percent SDS and 100µg/ml RNase A. Solid tissue can be dispersed by any appropriate device and lysis done using the same solution. Incubate 37 degrees Celsius for 60 minutes, then add Proteinase K to a final concentration of 0.2mg/ml. Continue the incubation at 37 degrees Celsius for two hours. Samples can remain in this state at room temperature overnight or extracted with Tris-saturated phenol (equal volume) and then a final extraction with chloroform. Phase separations are done by centrifugation at 2000 x g for five minutes at room temperature. Add a one-tenth volume of 2M sodium acetate pH 5.3 and two volumes of ethanol prior to spooling of the genomic DNA.

— THOMAS WOOD

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Q2

How do you validate your real-time PCR assays?

One of the most important steps is to sit down with the student or PI and obtain an understanding of the goals of their particular experiment. This way we can recommend an appropriate design, technology and protocol that will ensure a high-quality experiment and enable adequate analysis.

Once primers are designed, we recommend starting out with PCR fragment analysis on a 2.5 percent or 3 percent gel in order to evaluate the product. As most real-time primer design programs will give a very short amplicon, it is important to distinguish between the amplicon and possible primer dimers. When using Sybr Green assays it is critical to execute a dissociation assay after amplification in order to validate single PCR product.

— MARK BAND

Once I have validated my RNA/cDNA sample I then run a serial dilution of my cDNA to validate the assay. I find that serial dilution of cDNA will reliably serially dilute the target cDNA, while a serial dilution of the RNA/cDNA would also include the limitation of the reverse transcription step which is unnecessary for this purpose.

As I don't use SYBR assays but only ready-to-use optimized ABI assays, I have no need to validate primers and probe concentrations.

— LAURENCE BUGEON

Typically I'm using qRT-PCR to validate microarray data. Validation of the qRT-PCR is usually through biological replicates (eight if possible) or through protein expression assays (western blot).

— CHRISTINA FERRELL

To be honest, we do not do too much in the way of

validation. Some of the assays we get from Applied Biosystems are pre-validated, but the rest we simply judge by efficiency via the standard curve and the relative intensity of the assay.

— RYAN SPISSLER

We have a pretty good handle on ballpark primer or primer/probe concentrations for our custom assays, so we start by evaluating the assays at those ballpark concentrations with positive and negative control templates to demonstrate specificity. We'll then adjust concentrations if necessary based on the results. We follow that with template titrations to establish the detection range of the assay as well as its efficiency. For Sybr Green-based assays, we also perform dissociation curves to ensure that we are obtaining a single product, and to assess the

Continued on page 21

Q3

What controls and replicates do you use in your real-time PCR assays?

No template controls and no RT controls will give an estimate of possible DNA or PCR product contamination. A minimum of three technical replicates, preferably more, per sample are recommended to control for well-to-well variation. Pipetting technique is a critical factor and one of the prominent sources of variation. If possible we recommend working with electronic pipettes and diluting samples so that transfer volumes are above 2 microliters.

— MARK BAND

When I use an assay for the first time I include a “no template” control, I run all my assays in triplicates. When in doubt with exon-intron boundary and the possibility of amplifying contaminating DNA I run a no RT control.

— LAURENCE BUGEON

I use all the typical controls: positive (cell line known to express or over-express the gene), negative (cell line that does not express the gene), no RT (reverse transcription protocol without adding the polymerase, the results of which give you the amount of contaminating genomic DNA), and housekeeping gene (a gene with similar levels of expression to the gene of interest that is not modulated by the treatment: typical HK genes are GAPDH for high expression or clophilin for low expression).

All samples are run in triplicate technical replicates and four biological replicates unless there is some question as to the extent of gene expression, then we need eight biological replicates.

— CHRISTINA FERRELL

Our control steps can vary greatly but our most common

are the use of an exogenous internal positive control, if we are using Taqman probes. If we are using a Sybr Green reporter, we use the standard curve results as a control for plate-to-plate variation. Also, we do everything in triplicate and allow for one to be an outlier.

— RYAN SPRISLER

We always include “no template” controls in each assay. Ideally, we will also have positive and negative control samples as well. We run triplicates at a minimum.

— JOHN TINE

We recommend a minimum of three replicates per sample for both target and normalizer. While all samples analyzed for RNA expression are initially tested for DNA contamination, we routinely include a minus RT sample. No template controls are also included for each assay.

— THOMAS WOOD

Q4

How do you determine the efficiency of your real-time PCR assays? What efficiency is acceptable?

As part of the initial setup process a dilution series must be run to determine PCR efficiencies and the range of cDNA concentrations that will give accurate results. Efficiencies can be determined by plotting the Ct value against the log of dilution concentrations. The slope of this plot should be close to -3.3; however, the critical point is to have equivalent efficiencies between target and control genes.

— MARK BAND

For this I actually follow ABI recommendations as I am using their assays. I have found that running a serial dilution of my RNA for RT followed by a qPCR of a set amount of cDNA is very important here. This determines the range of RNA/cDNA for which the assay will be efficient. I then know where my efficiency is lost (copy number too high for reliable RT or

copy number too low for detection, RT efficiency, etc). Researchers often underestimate the importance of the RT step in their experiment and only assess the final qPCR step.

— LAURENCE BUGEON

Efficiency is determined using standard curves and a resultant calculation of percentage. Standard curves are created using 10-fold serial dilutions of bacterial vectors in which a known amount of the gene of interest has been incorporated. From this curve, efficiency is calculated using the formula: $[(10^{-1/\text{slope}(-3.23)} - 1)] * 100$. If one is using a positive control to make the standard curve, then the results must be expressed in terms of that positive control, i.e. dilution or cycle number.

When comparing gene expressions, both genes must have greater than 88 percent efficiency and

be within five percent of one another.

— CHRISTINA FERRELL

We always generate the efficiency of the primers using a standard curve from a one-tenth or one-half dilution series, and my rule of thumb has always been to have an efficiency of over 80 percent but to also have the products being compared within 10 percent of each other (i.e. experimental vs. housekeeping).

— RYAN SPRISLER

We determine efficiency by performing template titrations, usually 10-fold serial dilutions. The type of assay in part determines our

Continued on page 21

“Generate the efficiency of the primers using a standard curve.”

— Ryan Sprissler



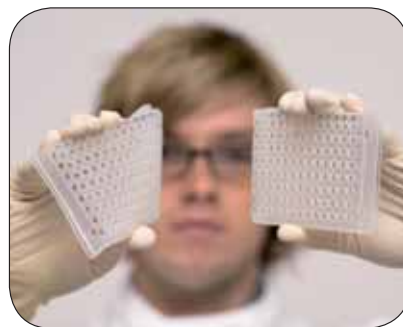
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Q5

How do you quantify your results? How do you generate your standard curve and/or $\Delta\Delta\text{CT}$?

Quantitation can be determined either by using a standard curve or, if reaction efficiencies are similar, by the relative change methods. I recommend running standard curves for most plates. This controls for plate variation. In addition, as most of the assays run in our lab are for organisms without commercially validated primer probe sets, it is often difficult to adjust for non-uniform efficiencies.

— MARK BAND

I use $\Delta\Delta\text{CT}$ after validating my assays in parallel and following ABI guidelines.

— LAURENCE BUGEON

Quantitation for absolute expression requires standard curves and results in: number of copies of gene of interest per copy of a housekeeping gene. The threshold must be the same for both genes (in the expo-

nential (linear) phase of the amplification) and the efficiency must be within acceptable limits.

Most clients use the ΔCt method: $2^{-(\text{Ave Cts for gene of interest}/\text{Ave Cts for Housekeeping gene})}$; this generates a decimal value representing the relative expression (copies of gene/copy of HK gene) without revealing actual copy number. The relative expression is normalized to the housekeeping gene.

$\Delta\Delta\text{CT}$ is actually a comparison of normalized data between two genes of interest. $2^{-[(\text{Ct Gene 1}/\text{Ct HK1})/(\text{Ct Gene 2}/\text{Ct HK2})]}$

In this case: the results are a decimal value for Gene 1 per copy of Gene 2.

— CHRISTINA FERRELL

All of our results have been relative quantitation represented in fold changes of regulation; we have never really used a copy number standard curve to give truly

quantitative results. For analysis I almost always use the $\Delta\Delta\text{CT}$ method and very, very rarely use the Pfaffl method which uses an efficiency correction calculation. In fact, I teach a class for graduate students where I don't even tell them there is an efficiency correction calculation in hopes they'll never use it.

— RYAN SPRISLER

Continued on page 21

“Quantitation for absolute expression requires standard curves and results in: number of copies of gene of interest per copy of a housekeeping gene.”

— Christina Ferrell

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Q6

Do you use normalizing genes? How do you choose them?

Normalizing genes are the standard for compensating for technical variations. It can often be very difficult to choose a gene that one can confidently show to be expressed at a consistent level among different conditions. Very often RT-PCR is used for validation of microarray results. In this case, the data from the microarray experiment might be used to choose a normalizing gene or set of genes. Depending on your budget, multiple genes can be used as normalizers, which would buffer variation of any specific gene. Another option is to use spiking controls. In this case it is critical to have accurate quantification of RNA concentrations of both the sample and spike transcripts.

— MARK BAND

Normalizing will depend on

the experiment and cell type. For the human or mouse genome there are 30,000 potential normalizers! A lot of scientists use qPCR to validate microarray experiments, so I suggest they check the behavior of the normalizer they were considering using in the microarray. I routinely use 18S, bearing in mind that due to the high copy number of 18S one must reverse transcribe only small amounts of RNA for reliable normalization. I found that 100ng is max in my system. Once again a serial dilution of the RNA will efficiently determine those values.

— LAURENCE BUGEON

The gene must be present in all samples in nearly the same quantity.

The gene must not be modulated by the treatment conditions.

The gene must be measurable with similar efficiency to the gene of interest.

Please note that while many use ribosomal RNA as a housekeeping gene because of its ubiquitous presence and ease of measurement it is not an appropriate gene to use for the following reason:

Ribosomal RNA is expressed at 100,000 times

“Depending on your budget, multiple genes can be used as normalizers, which would buffer variation of any specific gene. Another option is to use spiking controls.”

— Mark Band

the amount that most genes of interest are expressed. Because of this high level of expression, small changes are difficult to quantitate. Such changes in this so-called housekeeping gene would artificially increase or decrease the relative expression of any gene of interest. To use rRNA, one would have to dilute the sample significantly just to keep the Ct within a linear range, thus prohibiting multiplexing for the other genes of interest within those wells.

Many housekeeping genes must be tested before settling on one. There are many research groups that choose to use three or more housekeeping candidates and use the average expression for these three as the normalizing factor – most of my clients do not have enough sample to splurge in this way.

— CHRISTINA FERRELL

We always use housekeeping genes. My suggestion to our core users is to always pick three housekeeping genes and to average them to

“Generally, we also try to pick genes that have a relatively close level of overall expression to our gene of interest.”

— Ryan Sprissler

provide greater normalizing power. We obviously try to pick genes that show no difference in expression between the experimental and control conditions. We usually run a few experiments to see if we can detect any expression differences. Generally, we also try to pick genes that have a relatively close level of overall expression to our gene of interest. This is to say if the gene we are looking at is a low expresser, we would want to pick a normalizing gene that was also a low expresser as opposed to something beta actin or GAPDH.

— RYAN SPRISLER

Yes, we utilize normalizing genes when performing relative quantification. We

use GAPDH and beta actin, although it is important to verify that they are in fact not regulated in the particular experimental system in which one is working. They are often times not appropriate. When we are verifying microarray results, we have selected invariant genes from the array results to use as normalizing genes.

— JOHN TINE

Yes, we use a normalizer. While the client can specify the gene to be used, we recommend 18S or GAPDH. Polymerase beta has been used in select human studies. 18S has an abundance issue but generally has limited impact by the experimental protocol. We find some variance with actin that has prompted us to discourage its use.

— THOMAS WOOD

“While the client can specify the gene to be used, we recommend 18S or GAPDH.”

— Thomas Wood



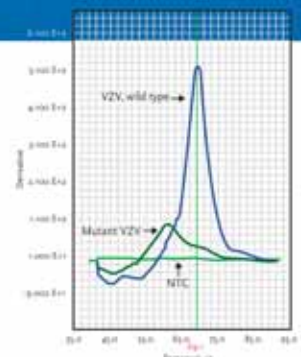
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Example melt curve showing MGB Alert technology detects sequence mismatches.

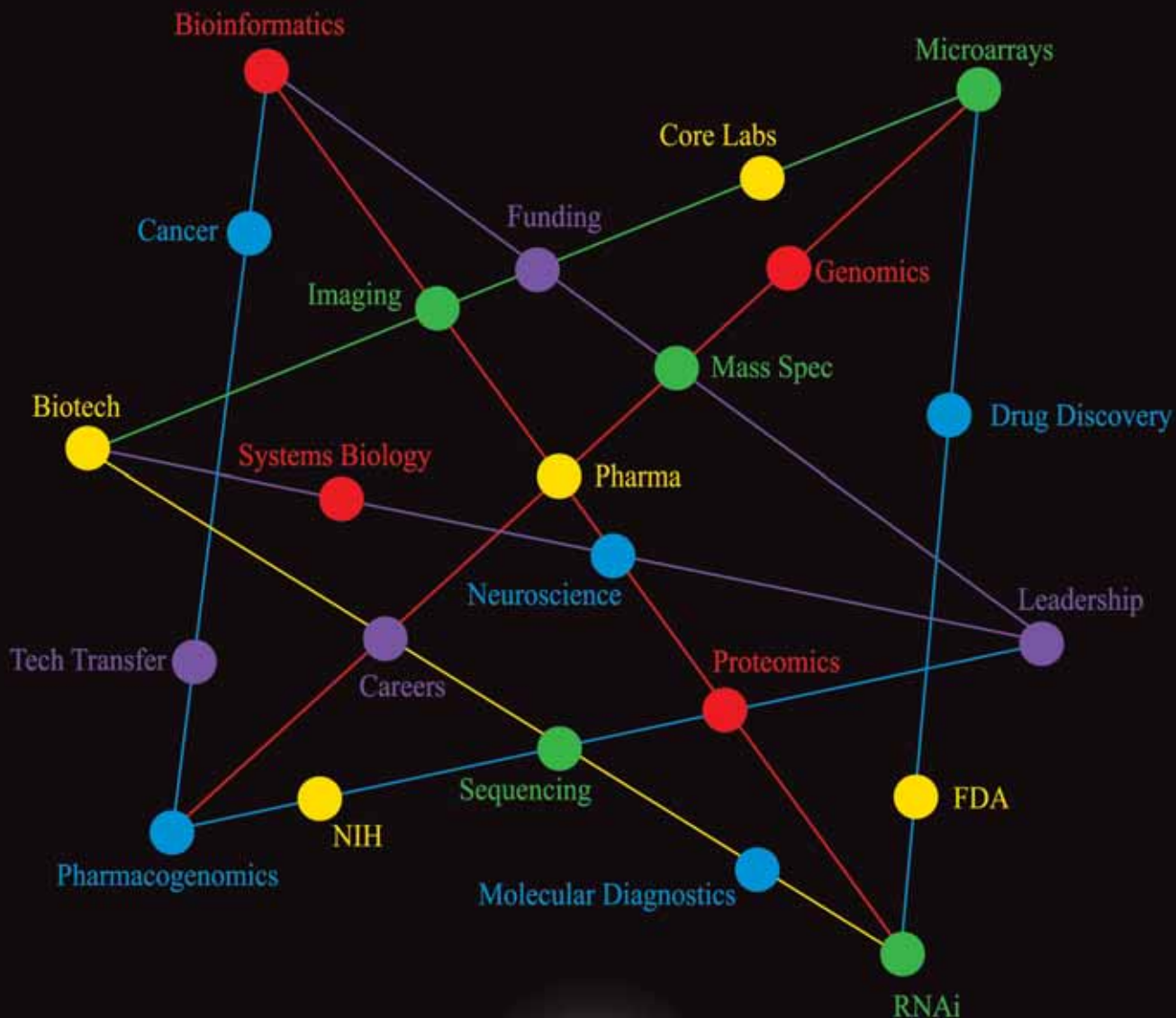


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development of primer-dimers in negative controls.

— JOHN TINE

Most of our requests are received from clients who are using real time to validate their gene expression data (Affymetrix Gene Chip or microarray-based). However, the most frequent data that I have seen for confirmation of an autonomous RT-PCR

assay has utilized western blot analysis.

When we design an assay for a client, especially RT-PCR based, we take the gene organization into consideration. I think it's a common practice to work across an intron in designing primers for real-time assays. However, not all species have been sequenced and some systems, like chicken, are incomplete. In many cases

this can be resolved by extrapolation of the gene organization in another system. Confirming the specificity of your amplicon for the target domain sequence by a Blast search is essential. We also like to validate the specificity of the assay by a functional test, ideally on a sample that has been shown to express the desired target RNA.

— THOMAS WOOD

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efficiency requirements. For a relative quantification experiment using the $\Delta\Delta C_t$ method, it is crucial that the efficiencies of the assays for each gene be similar. For an absolute

quantification where a standard curve is utilized, this is of less importance. As far as what is acceptable, I'm not comfortable with efficiencies below 98 percent for relative quantification.

— JOHN TINE

While we can detect 10 molecules of target, our service flyer states that we have a linear response to 25 molecules. These assays were done using synthetic transcripts.

— THOMAS WOOD

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When performing relative quantification, we use the $\Delta\Delta C_t$ method and rely on our instrument software to make the calculations (Applied Biosystems 7900HT). If our experiments are large enough to require multiple plates, we always include the calibrator sample on each plate. For absolute quantification,

we've used pooled cDNA, purified PCR products, and linearized plasmids as templates for constructing our standard curves. There are pros and cons to the use of each of these as templates. We rely on our instrument software to calculate the concentrations of our experimental samples.

— JOHN TINE

Absolute assays are performed with a standard curve that is generated using synthetic RNA (*in vitro* transcription using T7-based expression system). Relative assays utilize a $\Delta\Delta C_t$ approach. The client has the option of selecting the reference sample to be used in the analysis.

— THOMAS WOOD

List of resources

Sometimes even our esteemed experts haven't addressed your PCR question. Here's a list of resources that may help you solve your technical problem.

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Yuan JS, Reed A, Chen F, Stewart CN, Jr. (2006). **Statistical analysis of real-time PCR data.** *BMC Bioinformatics. 7: 85.*

CONFERENCES

Quantitative PCR Techniques Course

Avans University
Breda, Netherlands
April 9-11, 2008

Quantitative PCR

Cambridge Healthtech Institute
San Diego, California
April 20-23, 2008

qPCR Applications Workshop

TATAA Biocenter
Freising, Germany
July 7-11, 2008

Advances in qPCR

Select BioSciences
Stockholm, Sweden
September 17-18, 2008

PCR Perfection: Insider Tricks

Euroscicon
Hertfordshire, United Kingdom
October 3, 2008

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