A troubleshooting guide: Real-time PCR experts share their advice for handling your top experimental challenges

Real-Time PCR Tech Guide volume one

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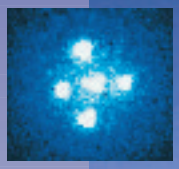

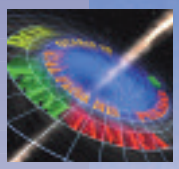

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A Match Made in Heaven

Letter from the editor

Welcome to the inaugural issue of *Genome Technology*'s reference guide series. This slim volume contains our first installment of answers to quantitative PCR problems.

Despite its youth, quantitative PCR has become ubiquitous in any lab dealing in nucleic acid quantification. The technology is conceptually simple, easy to use, and widely applicable. But these virtues belie a host of challenges, from improving assay design to working with degraded samples. These topics and more are addressed by our international panel of experts. Their field-tested tips are gathered here to help you troubleshoot your way through any experimental scenario involving real-time PCR. The guide itself is user-friendly, sleek, and complete with a resource list based on expert advice highlighted in the text. In short, it's made to keep on the bench, preferably within easy reach of your lab's qPCR work station.

This guide owes its existence to the generosity of our contributors, whose time, expertise, and counsel have transformed our initial questions into an accessible technical manual. With special thanks to them, we hope you enjoy the following pages.

— Jennifer Crebs

Index of experts

Pamela "Scottie" Adams
Director, Molecular Biology Core Facility
Trudeau Institute

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Associate Professor
Molecular Diagnostic Laboratory
Department of Clinical Biochemistry
Aarhus University Hospital

Vladimir Benes
Head, Genomics Core Facility, Scientific Core Facilities, Services & Technologies Unit
European Molecular Biology Laboratory

Stephen Bustin
Professor of Molecular Science
Queen Mary University of London

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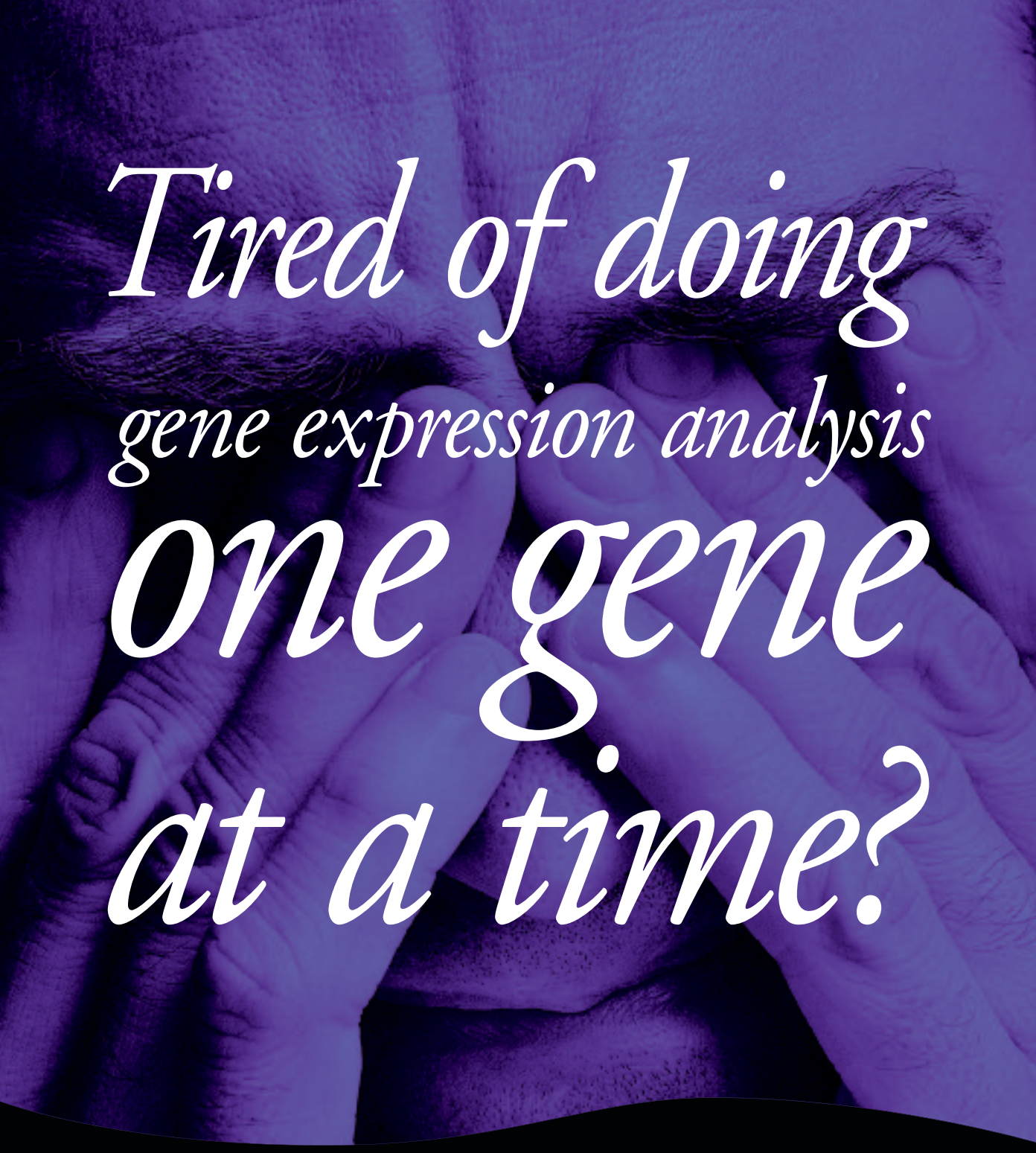
Vanderbilt University

Jo Vandesompele
Center for Medical Genetics
Ghent University Hospital

Michael Zianni
Manager/Research Associate
Plant-Microbe Genomics Facility
Ohio State University



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How do you select and validate normalization factors?

We test a panel of normalization genes — GAPDH, β-actin, β 2-microglobulin, GUS, and HPRT — to convince ourselves that expression is stable in the tissues and conditions we are using. GAPDH appears to be very stable in the tissues that we examine during various infections in the mouse. GAPDH is also useful because it can be used as an indication of genomic DNA contamination when used as a no transcription (-RT) control.

— Pamela Adams

Selection of candidates: I choose a set of candidate genes, typically five to 10, which I expect not to show expression variation under the conditions in the given experiment. Optimally the genes are selected based on some form of evidence (e.g. microarray data), but this is not a prerequisite (it will most likely increase the chance of identifying a good stabile expressed gene among the candidates).

Validation of expression stability: I then purchase or design real-time RT-PCR assays to these genes and test these on samples identical to, or at least very similar to, the samples in the given experiment. For clinical samples I have a minimum of eight samples per sample group. I recommend that the generated C_t values are transferred to linear values using standard curves. If standard curves are not applied, caution should be taken to verify equal amplification efficiencies of all assays; alternatively, measures should be taken to correct for differences in amplification efficiencies.

I then estimate the intra- and inter-group expression variation of the individual assays using the free-ware **NormFinder**. Finally, I select the candidate with the least expression variation as my normalization gene.

Alternatively, one can use a combination of two to three of the candidates to form a normalization factor — make sure that the candidates included have opposite directed inter-group variation. Otherwise the normalization factor is no better than using the candidates individually!

— Claus Andersen

Microarray data represent a treasure trove of reference genes for organisms where such data are available. Papers out there talk about genes expressed in normal tissues, and for some people that it is a very useful clue, but there are people who are working with organisms that are not so well characterized by microarrays. For these people, there is only testing, going through the literature, and more testing. Testing requires first selecting panels of reference genes: run them in a few situations, see if they are stable. This can be cumbersome, and there is the trouble that people are prone to compromise C_t difference, although they really shouldn't. This is a real issue that will need debate.

Some people share [Jo Vandesompele's] view regarding normalization factors, but it must be remembered that there is a difference between the ideal world and the world of daily research. There will be no such thing as a general set of reference genes. People need to be open minded and look to other biochemical pathways that are unrelated to the process they are ***(continued on page 9)***

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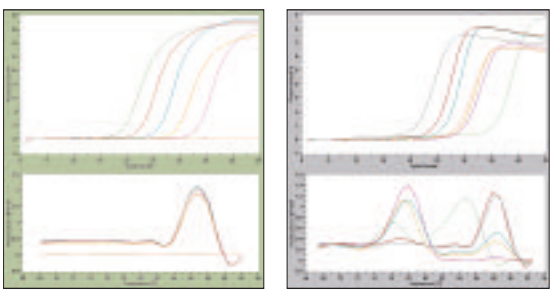

Performance of SYBR® Premix *Ex Taq*™ (Perfect Real Time) (left panel) vs. Roche's Fast Start DNA Master SYBR® Green I (right panel) using a Roche LightCycler®. Takara's SYBR® Premix *Ex Taq*™ demonstrates an excellent melting curve, particularly when low copy number templates are amplified. For more data demonstrating SYBR® Premix *Ex Taq*™ performance on other instruments, please see our website at **www.takaramirusbio.com**.

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How do you select and validate normalization factors?

(continued from p.7)

studying. Unfortunately, we are experiencing sort of a legacy issue with GAPD, actin, 18S genes, etc., because these genes were used at the beginning and now everyone is set to use them even when there is a lot of evidence out there showing that those genes can also be heavily regulated. Microarrays provide much richer data in this regard.

— Vladimir Benes

We have validated the use of total RNA in our particular experimental system (the colon) and express copy numbers per microgram total RNA. We have also developed a genomic DNA-based normalization method for RNA extracted from tiny samples, such as laser capture microdissected tissue. This uses Larry Wangh's PurAmp to lyse the tissue and perform the RT-PCR assay without having to purify the RNA any further (Hartshorn *et al.*, 2003).

— Stephen Bustin

We always test a large number of candidate reference genes, for example, using our endogenous control gene panel on representative samples, and then we select those with the least internal variation as references for the particular system. The latter selection can be done using, for example, **geNorm** or **GenEx**.

— Mikael Kubista

> "People are prone to compromise C_t difference, although they really shouldn't. This is a real issue that will need debate." *— Vladimir Benes*

We either use several factors or genes for normalization to maximize the likelihood that one or more of the factors are appropriate, or experiments are run to empirically determine which available factors will be useful for the experiments at hand. In situations where RNA quality may be a challenge, we use the latter approach. This allows us to test several samples and the normalization factors at the same time.

— Shawn Levy

According to our own established **geNorm** concept, we initially evaluate the expression stability of 10 candidate reference genes belonging to different functional and abundance classes. Using the geNorm software, we select the optimal number (at least three) of most stably expressed reference genes and use the geometric mean of these reference genes as an accurate normalization factor.

— Jo Vandesompele

The customers select the standardization genes and then do a thorough optimization protocol and test to determine whether the C_t values are changing for a given gene across the samples. I also encourage them to use PCR products or cloned genes to check efficiency.

— Michael Zianni

Assays That Play Well Together

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Anyone can design a Real Time PCR assay that 'plays alone.' The real challenge is developing multiplex assays where all components play well together. Bi-plexing a reference gene as an internal control with a target assay allows correction for sample to sample variation. Bi-plexing cannot be an afterthought however - it must be designed into the individual assays. Nanogen's MGB Eclipse™ By Design Service offers:

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Q2 What are your tips for improving assay design?

I like to design three sets of primers for each assay, test them using a SYBR green assay, and then use the best set with a probe in a Taqman assay. If you design primers to span an intron to eliminate genomic DNA amplification, be sure to check the size of the intron. Some introns are small and if the amplicon amplified from the genomic DNA is only 100 bp bigger than an amplicon from mRNA, it could have an effect on the accuracy of your assay. Search the literature carefully to be aware of alternate splicing scenarios and what effect they might have on the assay.

— Pamela Adams

If possible, change the exon-intron boundary.

Redesign primers — be careful to avoid polymorphic and unspecific primer targets.

Instead of pairing primers based on melting temperature, primers should be paired according to priming efficiency (can be estimated using **OLIGO Primer Analysis Software**).

— Claus Andersen

I would say not to be greedy. These days, the price of oligos has dropped to point where it is not an issue to have two to three primer pairs in one or even different regions of transcribed sequence, which can be tested. One doesn't have to run a whole set of 96 reactions, but maybe a couple of reactions using each primer pair. In this way, you can proceed rather rapidly. The advantage of this approach is that if bad goes to worst, primers can be combined within primer pairs. Basically, we try to get nested primers if possible, so if one pair is not working, we can take the other primer reversed from the amplicon to see if there has been an improvement.

There is this one thing which I have used recently by Premier Biosoft: they have software packages for microarray data, and **Beacon Designer** is the program for looking at secondary structures in the template when designing primers and probes. This software is a very nice tool, and there are also some Web pages where one can do similar things with the way sequences can fold.

— Vladimir Benes

Strive for maximum amplification efficiency; keep amplicons as short as possible; use specific primers where possible, otherwise oligo-dT; run standard curves with each assay; always run duplicate, no template controls, one set with tubes sealed prior to dispensing template, the other at the end of the dispensing. Always study amplification plots.

— Stephen Bustin

Use Evolution Operation (EVOP) optimization. If the assay is still not good enough, then change primers. For important assays we always test several primer combinations.

— Mikael Kubista

Have a good understanding of what you are designing and what the important considerations are. Like most things, the more you understand the process and what you are designing, the better the results will be. Also, practice makes perfect, so the more design and *(continued on page 17)*

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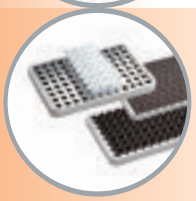

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Q3 How do you choose or develop standard protocols?

The protocol is dictated by the science. What question do you want to answer? I would search the literature for situations in which others have asked similar questions and see what protocols have been successful. Stephen Bustin's book, ***A-Z of Quantitative PCR***, is a wonderful resource and **Michael Pffafl's webpage** contains a wealth of information.

— Pamela Adams

Roughly, the qPCR protocol can be divided into three parts: isolation of specimen and total RNA from it, a reverse transcription step, and a quantitative PCR step. Nowadays, there is no doubt that the last step is perhaps the most straightforward from the three thanks to numerous high-quality kits available on the market. It is not to say that one shouldn't work carefully, but not so many things can go wrong here, provided that one is approaching it with the right attitude. It is the first two steps which can considerably influence the outcome of the experiment.

Basically, the protocol should be very robust in terms of performance. Although ideally one would like to have one protocol that fits all, there are groups of protocols that may be more applicable. That is, if you know that some amplicons of some RNAs are folding close to 3', in which case you cannot have amplicon designed there, then it is necessary to move to 5' end and adjust your strategy of priming the RT reaction. Ultimately, regardless whatever protocol has been chosen, the most important factor is consistency within the cohort of analyzed samples.

Preferentially, for any type of priming, the RT polymerase should be as thermostable and processive as possible. Mostly we are using SuperScript III and testing transcripts by Roche to see reproducibility over many runs. It is important to try to work with material that is as close to material one will get in a real experiment as possible. It is also better to use cDNA, not the plasmid, as the latter is too optimal. It is important to find the right measure, for too much of material can be inhibiting, for example.

We use a two-step method because the RT product can be archived; Stephen Bustin uses transcript-specific primers, but the product of this reaction cannot be used for assaying any other transcript, unfortunately.

Basically, people tend to think that qPCR is [an] easy method — it looks so deceivingly simple — but it really needs to be recognized that there are risks and caveats involved.

— Vladimir Benes

We have developed our own protocols:

(a) In our hands, the most reliable quantification of RNA is achieved using target-specific primers for the RT step. We empirically choose an optimal primer pair from four potential candidates for each target gene.

(b) We use Mfold to determine the best location for reverse (RT) primers and usually carry out the RT step at 60℃ for three minutes using *Tth* polymerase. We also use Mfold to determine the optimal position for the forward primer. ***(continued on page 17)***

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What do you do to achieve better primer specificity?

The best way to achieve primer specificity is by careful assay design. I prefer the extra assurance of specificity provided by the Taqman assay.

— Pamela Adams

Although it helps to Blast the primers to see if there is any secondary binding within transcriptome tested, people are not always working with organisms where sequence information is available. I think the ultimate test of your primers is a standard RT-PCR and checking its outcome on the high-percentage agarose gel. If the result is satisfying, we're running a small sample set on [the] qPCR instrument with SYBR Green and look at the dissociation curve. If the primer pair passes this test, then it's suitable for work.

— Vladimir Benes

Take nucleic acid secondary structure into account, perform the RT step at 60°C, reduce primer concentration as much as possible, and if all else fails, reverse transcribe from mRNA.

— Stephen Bustin

We don't experience primer specificity to be a problem, but poorly designed primers may give rise to primer-dimer products. This is minimized by proper primer design including avoiding any 3′ complementarity, using low primer concentrations, hot-start, and short annealing times.

— Mikael Kubista

Primer specificity is best achieved through proper design of the assay. Trying to optimize conditions to get a sub-optimal primer set to perform at high efficiency is typically more problematic than doing a good design or using a validated design to start with.

— Shawn Levy

Limit GC content of the 3′ end of the primer (maximum 40 percent GC content for last five bases). After primer design, we routinely do a Blast specificity search using the 'search for short, nearly exact matches' option at **NCBI's Blast portal** and using both forward and reverse primer sequence separated by three N's as one input sequence.

For more difficult designs where there is less freedom to choose the sequence to amplify (e.g. SNP genotyping or splice variant quantification), we sometimes add agents to enhance specificity, such as DMSO, or increase annealing temperature. We also keep the $MgCl_2$ and primer concentrations limited (max 3.5 mM and 250 nM for SYBR Green I assays, respectively), as higher concentrations allow more mispriming.

— Jo Vandesompele

Most customers use SYBR Green and have limited budgets, so they use thermal cycle changes instead of ordering different primers. Most customers (1) raise the annealing temperature, (2) collect data at a temperature intermediate to extension and melting, and/or (3) use five to 10 seconds for annealing.

— Michael Zianni

Q5 How do you enable amplification from damaged or impure samples?

If your amplicon is really short, then you still can use the sample and get decent curves, even if the mRNA has been heavily degraded. However, cDNA can be obtained only by priming the RT reaction with random primers. It's always good to check using the **Agilent Bioanalyzer** to see the profile of the sample's total RNA.

One thing that has been introduced in the lab is an assay assessing the scale of degradation. In regard to this, I recommend the work of Tania Nolan and Stephen Bustin. They have introduced two assays that are useful. One is the 3′:5′ strategy, that is checking quality of the 3′ and 5′ ends of selected transcript by qPCR and look to see if there is any C_t curve difference. If the mRNA is intact, then you should get the same C_t values for both ends. Nolan and Bustin also invented a method to assess presence of inhibitors; nevertheless, people try to push their luck here.

So, if a sample is very precious, with no chance of recovery and such that you cannot apply any further purification steps, then the length of the amplicon is very important. If the issue is degradation, you cannot do oligo-dT priming, but are priming with random primers, it is important that it is consistent with all samples in a panel. In any case, there is always hope in these situations (of course, if it cannot be done, then sorry).

— Vladimir Benes

It is important to realize that results from impure/damaged RNA must be treated with caution. We screen our RNA using the **Agilent Bioanalyzer**, assess the 3′:5′ ratios of reference genes, and screen for inhibitors present in the sample.

— Stephen Bustin

When amplifying damaged samples we believe one should use specific RT primers, which are the same as one set of the PCR primers, to minimize variation in the RT step due to RNA degradation.

For complex samples it is important to account for matrix effects, which can be done either by *in situ* calibration (standard dilution) or standard addition (Stahlberg *et al.*, 2003).

— Mikael Kubista

RNA isolation procedures that are optimized for the conditions of the sample (frozen or FFPE, tissue or cell lines, etc.) make a tremendous difference in the quality of RNA. When the quality of the RNA cannot be made better, ensuring that all of the RNA that will be used in differential comparisons has the same overall quality is very important to ensure consistent and comparable results. If impurities are present, cleaning up the RNA samples prior to cDNA synthesis or cleaning up the cDNA material can be very helpful.

— Shawn Levy

We have experienced that it is fairly easy to obtain beautiful amplification plots for degraded samples. The important question, however, is how reliable data are coming from such samples. In a recent study, we demonstrated that expression patterns might vary in relation to the degradation status of

the samples (Perez-Novo *et al.*, 2005). In the same paper, we recommended not to analyze intact and degraded samples together.

With respect to working with impure samples, we try to address this issue by performing proper nucleic acid extraction, followed by extensive quality control, such as inspecting the UV absorption spectrum of the nucleic acids and performing capillary gel electrophoresis to assess the length of the ribosomal RNA molecules.

— Jo Vandesompele

Q2: What are your tips for improving assay design?

(continued from p.11)

testing, the better you get. Of course, with the number of vendors selling pre-validated assays, the easiest way to improve your assay design is to buy one rather than build one.

— Shawn Levy

Perform extensive in silico assay evaluation, using Blast specificity analysis of the primer pair, Mfold secondary structure prediction of the amplicon sequence (significant structures reduce amplification efficiency), search for SNPs present in the primer annealing region, and transcript variant analysis (alternative axons, etc.).

We are currently implementing a Web-based and freely available in silico assay evaluation analysis pipeline for user-designed assays. This module will be part of the next update of our real-time PCR primer and probe database (**RTPrimerDB**) and aims at streamlining assay quality prediction prior to experimental evaluation, based on above cited established in silico tools, such as Blast specificity search, Mfold secondary structure analysis, presence of SNPs or plain sequence errors, and graphical visualization of the aligned primer sequences on the target gene.

— Jo Vandesompele

Do a thorough optimization at the start focusing on annealing temperature, template concentration, primer concentration, and utilizing the best bench technique as possible while setting up the reactions. I make sure customers realize there are no shortcuts or magic bullets, and thorough optimization is critical.

— Michael Zianni

Q3: How do you choose or develop standard protocols?

(continued from p.13)

(c) A standard curve is developed for each target. We aim to achieve an amplification efficiency of more than 95 percent. If that is not possible, we aim to achieve consistent amplification efficiencies between runs, monitored using a standard curve as well as a positive control sample.

— Stephen Bustin

When using standard protocols, it is important that all assays are optimized under standard conditions. If this is not the case, matrix effects from biological samples will complicate interpretation seriously.

— Mikael Kubista

We start with recommended or established protocols and then make modifications as dictated by experimental needs or try optimizations to improve performance or efficiency. For the most part, we use established and well used protocols — usually from the manufacturer. If it isn't broken, don't fix it.

— Shawn Levy

Standard protocols or operating procedures are very important in order to achieve consistent and accurate results. As our lab was one of the early adopters of qPCR technology, we developed our own experimental protocols, from nucleic acid quality control, over DNase treatment, cDNA synthesis, PCR reaction setup, and data analysis. It is important to rigorously control the quality at each step and use both positive and negative controls to evaluate the reliability of the results.

— Jo Vandesompele

List of resources

In the preceding pages, our experts referred to a number of resources. We've compiled their recommendations in the following list. Whether you're a qPCR ace or apprentice, these Web tools and publications are sure to be helpful.

Publications

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Agilent Bioanalyzer: http://www.chem.agilent.com/

GenEx: http://www.multid.se/GenEx/genex.htm/

Gene Quantification web page (edited by Michael W. Pfaffl): http://www.gene-quantification.info/

GeNorm: http://medgen.ugent.be/~jvdesomp/genorm/

NCBI's Basic Local Alignment Search Tool:
http://www.ncbi.nlm.nih.gov/blast/

NormFinder:
http://www.mdl.dk/publicationsnormfinder.htm/

OLIGO Primer Analysis Software:
http://www.oligo.net/

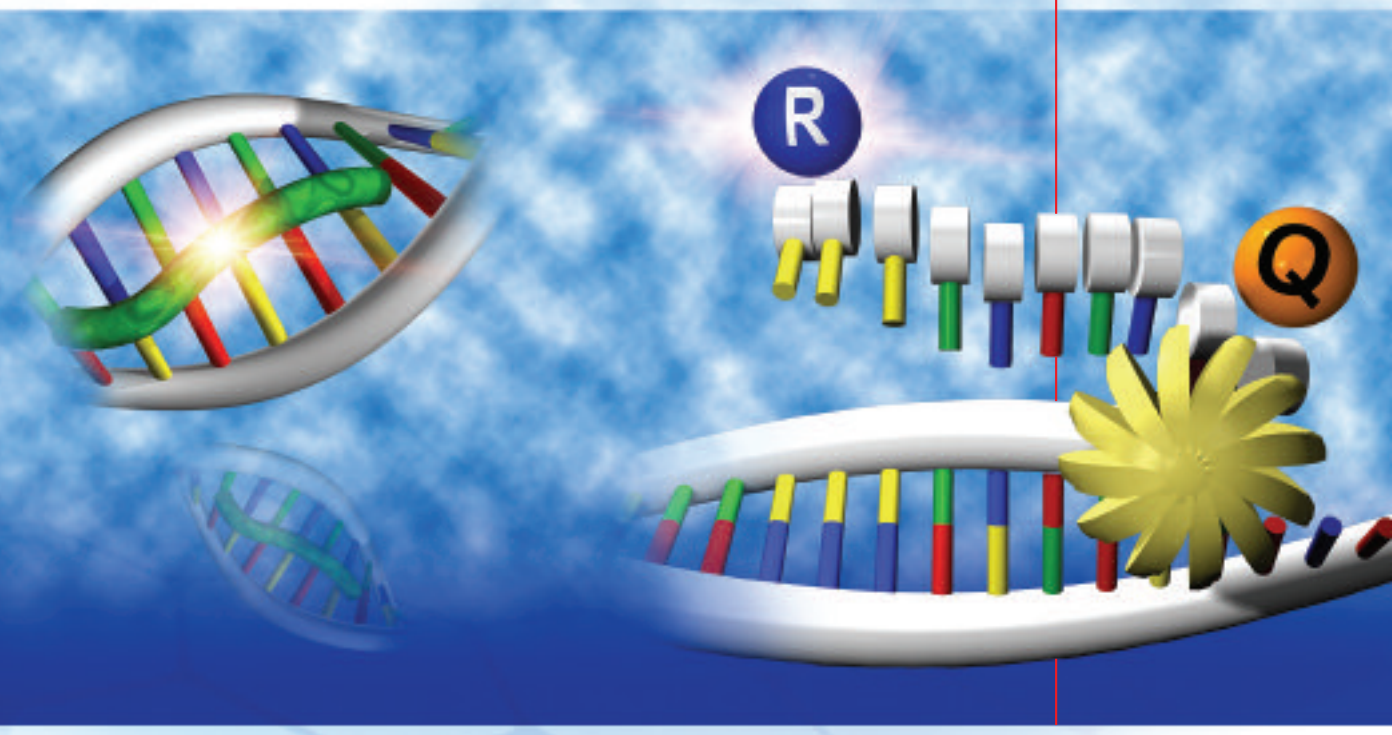
Premier Biosoft Beacon Designer:
http://www.premierbiosoft.com/molecular_beacons/

Real Time PCR Primer and Probe Database (RTPrimerDB): http://medgen.ugent.be/rtprimerdb/

TATAA Biocenter: http://www.tataa.com

Optimized, Flexible, Sensitive...

We Have the Right QPCR Reagent for You.



Sigma's broad range of QPCR reagents provide the flexibility needed to meet your QPCR needs. From probe based to SYBR® Green dye based QPCR, our products are compatible with all instruments and optimized to help you achieve successful results.

- Optimized ReadyMixes™ let you choose the detection method.
- Compatible with tube or plate based instruments.
- Greater sensitivity, efficiency, and linear response.

Probe based Quantitative PCR	
Product Number	Product Name
D6442	JumpStart™ Taq ReadyMix for High Throughput Quantitative PCR (blended with Reference dye)
D7440	JumpStart Taq ReadyMix for Quantitative PCR
D9191	JumpStart Taq ReadyMix with dUTP for Quantitative PCR
SYBR Green based Quantitative PCR	
Product Number	Product Name
S9194	SYBR Green JumpStart Taq ReadyMix for High Throughput Quantitative PCR (blended with Reference dye)
S4438	SYBR Green JumpStart Taq ReadyMix for Quantitative PCR
S5193	SYBR Green JumpStart Taq ReadyMix without Magnesium Chloride
Capillary based Quantitative PCR	
Product Number	Product Name
S1816	SYBR Green JumpStart Taq ReadyMix for Quantitative PCR, Capillary Formulation
D9191	JumpStart Taq ReadyMix with dUTP for Quantitative PCR
Quantitative RT-PCR	
Product Number	Product Name
QR0100	SYBR Green Quantitative RT-PCR Kit
QR0200	Quantitiave RT-PCR ReadyMix for probe based applications

For more information and to evaluate Sigma's Quantitative PCR products, contact us at: **sigma-aldrich.com/qpcr1** *or call* **1-800-325-3010**

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What do all these images have in common? The answer is 5. Introducing the iQ5 real-time PCR detection system and reagents, bringing true five-color detection capabilities to all of your real-time PCR applications.

Five reasons why the iQ5 is the ultimate system for value and flexibility:

- **Powerful thermal gradient**
- **Optical detection system features user-changeable filter sets**
- **Gene expression analysis by relative quantity (ΔC_T) or normalized expression ($\Delta\Delta C_T$)**
- **Customizable software reports that offer comprehensive summaries of assay run conditions, data graphs and tables, and data analysis parameters**
- **Optimized multiplex reagents: increase throughput, control costs, and maximize your data output with the new iQ multiplex powermix**

iQ5 real-time PCR detection system and iQ™ multiplex powermix

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