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Gene quantification using real-time quantitative PCR: An emerging technology hits the mainstream

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The recent flood of reports using real-time Q-PCR testifies to the transformation of this technology from an experimental tool into the scientific mainstream. Many of the applications of real-time Q-PCR include measuring mRNA expression levels, DNA copy number, transgene copy number and expression analysis, allelic discrimination, and measuring viral titers. The range of applications of real-time Q-PCR is immense and has been fueled in part by the proliferation of lower-cost instrumentation and reagents. Successful application of real-time Q-PCR is not trivial. However, this review will help guide the reader through the variables that can limit the usefulness of this technology. Careful consideration of the assay design, template preparation, and analytical methods are essential for accurate gene quantification. **© 2002 International Society for Experimental Hematology. Published by Elsevier Science Inc.**

A literature search with the keyword "quantitative PCR (Q-PCR)" or "real-time PCR" will generate literally thousands of hits, testifying to the emergence of this technology into the mainstream of many scientific disciplines. If the same search were to have been performed a few years ago, only a couple of hundred hits would have been returned. What has accounted for this sudden increase of papers utilizing this technology? The first documentation of real-time PCR was in 1993 [1], and yet this technology has only recently hit the mainstream. Perhaps the primary reason for this was the great expense of the instruments, but it has certainly been exacerbated by the complexities of performing reproducible real-time Q-PCR studies, and real-time PCR has only recently been widely accepted as a valuable technique. With the increasing number of real-time PCR thermocyclers on the market and the decreasing prices of these instruments, as well as the reagents, many more people now have access to this technology. In order to design and analyze experiments using real-time PCR it is not sufficient to simply extend one's knowledge of standard PCR, or even semiquantitative PCR. Many more controls are needed in order to be certain of results when using real-time PCR, as it differs significantly from simply looking for a band on a gel.

The PCR reaction generates copies of a DNA template in an exponential fashion. Due to inhibitors of the polymerase reaction found with the template, reagent limitation, or accumulation of pyrophosphate molecules, eventually the PCR reaction is no longer generating template at an exponential rate (otherwise known as the "plateau phase"), and some reactions will generate more product than others. This is the most important reason that end-point quantitation of PCR products is so unreliable. With the ability to measure the PCR products as they are accumulating, or in "real time," it is possible to measure the amount of PCR product at a point in which the reaction is still in the exponential range. It is only during this exponential phase of the PCR reaction that it is possible to extrapolate back to determine the starting amount of template. During the exponential phase in real-time PCR experiments a fluorescence signal threshold is determined at which point all samples can be compared. This threshold is calculated as a function of the amount of background fluorescence and is plotted at a point in which the signal generated from a sample is significantly greater than background fluorescence. Therefore, the fractional number of PCR cycles required to generate enough fluorescent signal to reach this threshold is defined as the cycle threshold, or Ct. These Ct values are directly proportionate to the amount of starting template and are the basis

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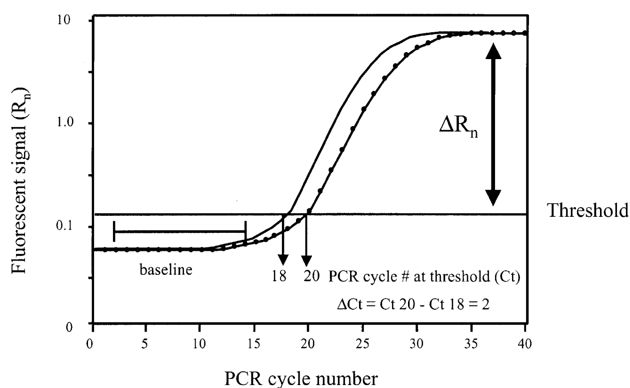

Figure 1. A hypothetical amplification plot illustrating the nomenclature typically used in real-time Q-PCR experiments. The amplification plot is the plot of fluorescence signal vs PCR cycle number. The baseline is defined as the PCR cycles in which a signal is accumulating but is beneath the limits of detection of the instrument. The signal measured during these PCR cycles is used to plot the threshold. The threshold is calculated as 10 times the standard deviation of the average signal of the baseline fluorescent signal. A fluorescent signal that is detected above the threshold is considered a real signal that can be used to define the threshold cycle (Ct) for a sample. The Ct is defined as the fractional PCR cycle number at which the fluorescent signal is greater than the minimal detection level. The Ct values of different samples are then used to calculate the relative abundance of template for each sample. In this plot the solid line crosses the threshold at PCR cycle number 18 whereas the dotted line crosses at 20. By subtracting 18 from 20, there is a two-cycle difference between these two samples or a ΔCt of 2. Due to the exponential nature of PCR the ΔCt is converted to a linear form by $2^{-}(\Delta Ct)$ or fourfold difference. This calculation is used when performing a relative quantitation analytical method.

for calculating mRNA expression levels or DNA copy number measurements (Fig. 1).

What is real-time Q-PCR?

Real-time quantitative PCR is the reliable detection and measurement of products generated during each cycle of the polymerase chain reaction (PCR) process which are directly proportionate to the amount of template prior to the start of the PCR process. To accomplish this it is necessary to have a method of detecting the accumulation of PCR product and an instrument in which to perform the thermocycling that is adapted to record the results during each PCR cycle in real time. Early attempts to perform Q-PCR, prior to real-time instrument, relied on visualization of PCR products using intercalation of ethidium bromide (or other intercalating dyes) at an empirically determined PCR cycle number. These products were run on standard agarose gels and quantified with radio-imaging or other densitometric means. The addition of competitive PCR reactions provided some increase in the quantitative capacity, but together these methods fell short of being convenient, robust, and reliable quantitative assays.

The first reported method of real-time PCR, by Higuchi in 1993 [1], used ethidium bromide intercalation during the PCR process and a modified thermocycler to irradiate the samples with ultraviolet (UV) light and then detected the resulting fluorescent signal with a charged coupled devise (CCD) camera. The fluorescent signal was plotted as a function of cycle number. The resulting plot, now a very familiar sight (Fig. 1), gave a good indication of the amount of PCR product that was generated during each cycle of PCR (except for those early cycles that are beneath the detection limits of the CCD camera). The primary drawback to this approach, other than the use of a carcinogen like ethidium bromide, is that nonspecific PCR products are equally detected and included in the total amount of fluorescent signal measured.

Today, the chemistries most commonly employed include 5′ nuclease assays using TaqMan probes [2,3], molecular beacons [4], and SYBR Green I intercalating dyes [5,6] (Fig. 2). Other methods have also been reported [7,8]; however, in all cases a fluorescent signal is generated during the PCR process that is captured by any one of several different real-time instruments. Due to added specificity, the addition of a hybridization probe makes the real-time Q-PCR assay much more robust. The 5′ nuclease assay generates a fluorescent signal by cleavage of a fluorescent molecule on the 5′ end of a target specific oligonucleotide (TaqMan probe, Applied Biosystems, Foster City, CA, USA). In native form the 5′ fluor is quenched by a second molecule on the 3′ end of the probe. When excited by the light source, the intact probe emits a signal in a wavelength characteristic of the 3′ molecule due to Förster (or fluorescence) resonance energy transfer (FRET), which shifts the energy to be released as light or as heat when using a black hole quencher (BHQ; Biosearch Technologies, Novato, CA, USA). A novel type of TaqMan probe is available from Applied Biosystems which uses a "minor groove binding" (MGB) moiety on the 3′ end that raises the effective melting temperature (Tm) of the probe, thereby enabling the probe to be significantly shorter and enhancing the function in an allelic discrimination assay [9]. Moreover, these probes are capable of determining the expression level of a specific allele to the exclusion of the opposite allele that differs by only one nucleotide (unpublished data).

Currently there are numerous real-time thermocyclers on the market; these include the ABI7700, ABI7900, and ABI7000 (Applied Biosystems), MX4000 (Stratagene, LaJolla, CA, USA), Lightcycler (Roche, Alameda, CA, USA), iCycler (Bio-Rad, Hercules, CA, USA), Smartcycler (Cepheid, Sunnyvale, CA, USA), and the Robocycler (MJ Research, Incline Village, NV, USA). By far the most common and the first to reach the mass market was the AB7700, which has recently been replaced with the ABI7000 or ABI7900. One of the major advantages of the ABI instruments is the collection of data from a "passive reference" signal to normalize each reaction for variances in the optics of the system. The MX4000 can also compensate for this variance, has software that is easy to use, and is able to perform multiplex reactions, with up to three different PCR reactions in a single tube. Although it is beyond the scope of

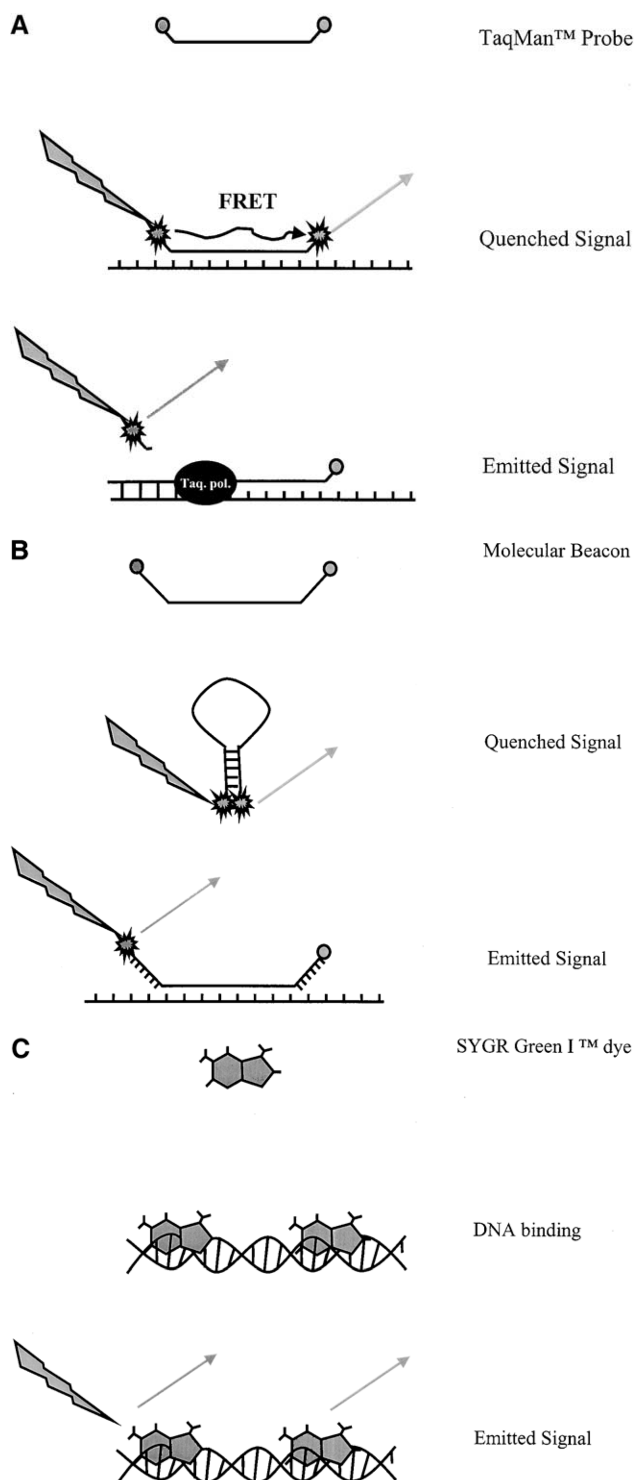

Figure 2. Methods used to generate a fluorescent signal in real-time Q-PCR experiments. Although other methods exist [8], these are the most commonly used in real-time Q-PCR studies. **(A)** A typical TaqMan probe used in a 5′ nuclease assay to generate a signal using the 5′ to 3′ exonuclease activity of Taq DNA polymerase. This probe is designed to hybridize to the template between the standard PCR primers. Once cleaved from the rest of the probe, the 5′ dye molecule is freed from the quenching effect (via FRET) when in close proximity to a quenching molecule such as TAMRA (6-carboxy tetramethyl rhodamine). The 5′ dye can be one of several different molecules but 6-FAM (6-carboxyfluorescein) is the most common one used. **(B)** A molecular beacon. This is similar to a TaqMan probe in that it uses quenching to prevent unwanted fluorescent signal, although through a direct transfer of energy. However, it differs in that it is not cleaved by the 5′ exonuclease activity and only generates signal when hybridized to the template. The addition of a hairpin is needed to provide the quenching effect. When hybridized, the distance of the 5′ dye molecule from the quenching molecule (usually DABCYL) is great enough that there is very minimal quenching of the signal. Data must be analyzed during the annealing phase of the PCR. **(C)** SYBR Green I DNA-binding dye. When using this method there is no need to design a third, modified oligonucleotide or hybridization probe. A fluorescent signal is only possible when the sample is excited by the light source when the dye is bound to the DNA molecule (possibly by intercalation in the minor groove of double-stranded DNA). This is the cheapest of the methods but suffers from the need to optimize the PCR reactions such that the primers do not form primer-dimers. SYBR Green I dye cannot discriminate between real template and artifact bands, unlike that of TaqMan probes or molecular beacons.

this review article to compare and contrast all the instruments available on the market, one must be careful when choosing which instrument to buy. All of these instruments are capable of performing real-time PCR, yet they are not all equal. Cost should not be the only factor when making a choice; the cheaper models cannot compensate for the variance in the optics and therefore are not capable of detecting smaller differences. The higher-throughput instrument (e.g., AB7900) may be more than you need; with a 384-well format, it is capable of running a vast number of allelic discrimination assays. Great care and research should be given to your choice; it is imperative to match the instruments capabilities with your needs.

General applications of Q-PCR

Applications of real-time Q-PCR are numerous. They include: mRNA expression studies, DNA copy number measurements in genomic [10–12] or viral DNAs [13], transgene copy number [14 and unpublished data], allelic discrimination assays [15,16] and confirmation of microarray data [17–19]. Some of the most recent applications, which demonstrate the sensitivity of this technology when applied to expression analysis of limited samples, include expression analysis of specific splice variants of genes [20] and laser capture of microdissected material [21]; paraffin-embedded tissues [22,23]; flow-sorted cells, including stem cells (unpublished data); or randomly amplified RNA from single cells [24].

Specific applications of real-time Q-PCR to experimental hematology

Real-time Q-PCR has been used in a small number of studies recently published in this journal [25–29]. However, given the recent proliferation of studies in which real-time Q-PCR has been used, it is only a matter of time before the full potential of its application to hematology in realized. Specific examples include measurement of translocation gene

products, DNA copy number measurements in leukemia, and analysis of mRNA expression levels of genes important for the differentiation of cell types of an immune response.

Detection of minimal residual disease

Hematological malignancies characterized by specific genetic translocations can be used as tumor markers while monitoring the response to therapy. Treatment options often involve chemotherapy, radiotherapy, and/or bone marrow transplants. Over recent decades these advances have greatly improved the chances for survival for these patients. However, the risk of recurrence remains a significant obstacle for complete remission. Thus, the detection of minimal residual disease is a crucial step towards further refining treatment regimens [29,30]. The use of real-time Q-PCR is becoming a necessary research tool for detecting the molecular events underlying these recurrences and may guide therapeutic decisions based on how individual patients respond at the molecular level. Thus quantitative measurements can be used to define correlations between the amount of fusion products and clinical outcome.

In a specific example, the reciprocal translocation t(8;21)(q22;q22), in which the gene for acute myeloblastic leukemia (AML)-1 transcription factor is fused with a gene (MTG8) on chromosome 8, is perhaps the most frequent chromosomal aberration resulting in AML. The disruption of normal transcriptional regulation by AML-1 is likely to result in leukemogenesis. Despite the positive outcome of treatment for many AML patients, a significant proportion will relapse. Moreover, even without relapse many patients will still test positive for an AML-1/MTG8 fusion transcript using standard PCR techniques. Perhaps limited by the detection of rare preleukemic cells, standard PCR techniques are not capable of stratifying patients, as the amount of fusion product may be a better prognostic indicator. Therefore, recent studies [31,32] have demonstrated the use of real-time Q-PCR for detecting fusion products that is helpful to identify those patients with quantifiable minimal residual disease and may prove valuable as a prognostic indicator or for the assessment of treatment options.

Similar studies have been conducted to quantify other translocation fusion transcripts, such as BCR-ABL in chronic myeloid leukemia (CML) [33,34 before and after allogeneic transplant [35, or to determine the response to treatment with interferon-α (INF-α) in CML patients [36]. Quantification of leukemia-specific TEL-AML1 fusion transcript levels in the prediction of relapse of childhood acute lymphoblastic leukemia (ALL) [37,38] or to measure minimal residual disease of ALL [39] is possible using Q-PCR methods. Furthermore, real-time Q-PCR has been used to quantify the chromosomal translocation t(14;18)(q32;q21) [40,41] and *bcl*-2 rearrangement in patients with follicular lymphoma (FL) [42] or stem-cell harvests used to treat patients with non-Hodgkin's lymphoma [26]. It was also used to measure the prevalence of *bcl*-2 rearrangements in the peripheral blood of normal individuals in order to determine how background levels of this rearrangement might impact the measurement of disease in patients with FL [43]. Many of these studies have benefited greatly by the application of real-time Q-PCR methods. With highly sensitive and quantitative Q-PCR assays available, stronger clinical correlations to molecular events may now be possible.

DNA copy number measurements in leukemia

DNA copy number measurements are important in determining the extent of genomic imbalance that underlies most malignancies. There are numerous techniques available for measuring DNA copy number in tumors; each method has specific advantages and disadvantages. Chromosomal CGH can detect imbalances across the entire genome, but at relatively low resolution. Fluorescent in situ hybridization (FISH) can provide copy number measurements in a cell-specific manner, but it is difficult to perform in high throughput and is difficult to count ∼25 or more DNA copies. PCR has been used for determining allelic imbalance (or loss of heterozygosity) using polymorphic simple-sequence repeats; however, if it is performed as an end-point PCR assay, quantitative conclusions can be misleading.

Real-time Q-PCR has been used in several studies [11,12,44,45] in which allelic imbalance is determined. Quantifying the DNA copy number of specific genes or markers at many different loci enumerated regions of chromosomal imbalance. In defining the quantitative microsatellite analysis (QuMA) technique, an animal model for leukemia was used to define the limits of a common chromosomal region lost from mouse chromosome 2, syntenic to regions of human chromosomes that are also associated with leukemia. In the SJL mouse strain exposed to ionizing radiation, chromosomal breakage occurs, including portions of chromosome 2, and many of these mice will go on to develop leukemia. Genes located in the deleted region are likely to be important for controlling cellular proliferation, and identification of these genes would help clarify the molecular events required to induce cancer in this model system, which in turn may shed light on the genes involved in leukemia patients. Since the region is quite large (∼23 cM) the possibility to measure many loci quickly using QuMA is helpful to further reduce the minimal region that is commonly lost in these mice.

Expression analysis of cytokines

The reliable quantitation of cytokine mRNA expression profiles is important for analyzing specific immune responses [46]. Accurate quantitation of cytokine levels can be helpful in measuring immunological responses. Cytokines play an important role in autoimmune, allergic, and infectious diseases as well as proinflammatory and antiinflammatory responses [47]. In addition, they are commonly the focus of studies of immunosuppressive therapy during

organ transplantation. Cytokines also play a modulatory role in the pathogenesis of chronic inflammatory diseases such as rheumatoid arthritis. Similar to cytokines, expression levels of chemokines and chemokine receptors in a wide variety of cell types are crucial to a better understanding of the spectrum of action of the chemokines [48]. The use of Q-PCR methods has greatly refined the role of cytokines and chemokines in a number of biological contexts.

Criteria for developing robust real-time Q-PCR assays

The best method to use for real-time Q-PCR studies depends on the scientific question being asked. The first dividing point is whether the study is focused on measuring mRNA expression levels (Q-RT-PCR) or DNA copy number. Other factors such as the number of genes and the number of samples to be screened will also influence the method of choice. Options for generating the fluorescent signal, primer design, and analytical methods critical to developing a robust real-time Q-PCR assay are described below.

Experimental Design

There are primarily two types of Q-PCR analysis: "relative quantitation" and "standard-curve quantitation"(see Applied Biosystems user bulletin #2, URL listed in Table 1). The latter has been termed "absolute quantitation" [49 (and others)]; however, this is a misnomer. A more appropriate term for this method is standard-curve quantitation, as a standard curve (fivefold or10-fold serial dilution) of "knowns" is used to quantify the "unknowns" of interest. The fact that this method relies on a set of knowns is the reason it cannot be "absolute." No matter what the source or how carefully it is measured, there is no way to know exactly how much or how many copies of a known template truly exists in a given well of a known sample.

Relative quantitation is the analytic method of choice for many real-time Q-PCR studies. In this method a comparison within a sample (DNA or cDNA) is made with the gene of interest to that of a control gene (Fig. 1). Quantitation is done relative to the control gene(s) by subtracting the cycle threshold, or Ct, of the control gene from the Ct of the gene of interest, and the resulting difference in cycle number (ΔCt) is the exponent of the base 2 (due to the doubling function of PCR), representing the fold difference of template for these two genes. Comparison to a control gene has both positive and negative connotations. The assumption must be made that the chosen control gene does not vary in copy number or expression level amongst the samples of study. If this assumption does hold true, then a lot more samples can be run on any given plate or multiple plates that will be completely comparable. With proper master mixing of reagents and the use of a positive displacement pipette, the amount of input template into each PCR well will be very similar. Variations in the triplicate measurements of each sample for each gene will indicate the amount of variance between genes for each sample.

Using the relative quantitation method requires that the PCR efficiencies of all genes be similar and preferably at or above 90%. This can be measured by performing a 10-fold serial dilution of a positive control template, and by plotting the Ct as a function of log[10] concentration of template (see Applied Biosystems user bulletin #2; Table 1); the slope of the resulting trend line will be a function of the PCR efficiency. A slope of -3.32 indicates the PCR reaction is 100% efficient (Fig. 3). Deviations from 100% efficiency can be calculated by putting the value for the slope (S) into the following equation: PCR efficiency $= (10^{(1/-s)}) - 1$. For more details on these and other calculations see [11,12,50 and Applied Biosystems user bulletin #2. The variables most likely to affect PCR efficiency are $MgCl_2$, primer, and probe concentrations. It is best to maintain a constant annealing temperature if possible, as all assays can be run under the same PCR cycling conditions.

The standard-curve quantitation method is successfully applied when measuring a small number of genes in either a

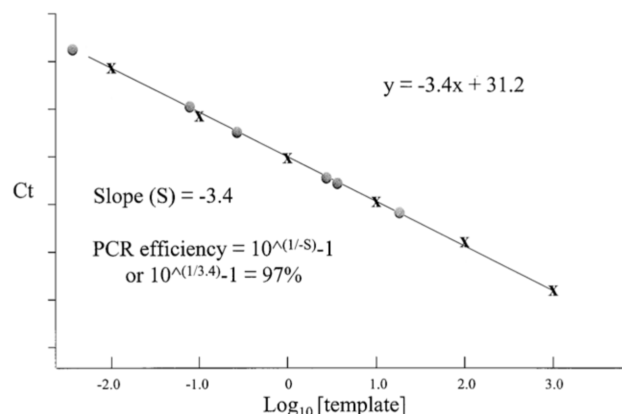

Figure 3. A hypothetical standard-curve plot for calculation of PCR efficiency and quantitation. Typically a fivefold or 10-fold serial dilution of a positive control template is used to generate the standard curve. The resulting Ct values for each input amount of template are plotted as a function of the log[10] concentration of input amounts (black X's) and a linear trendline is fit to the data. This is done both for optimizing a PCR reaction as measured by the PCR efficiency and for quantitation of unknown samples (gray dots). Determining PCR efficiency is especially important for relative quantitation. The resulting slope of the line fit to the data is used to determine the PCR efficiency as shown in the formula. An ideal slope should be -3.32 for 100% PCR efficiency; in this example it is 97%. The function that defines this slope is also used to calculate the amount of unknown samples. Most real-time PCR instruments have software that can automatically compute the amount of template of an unknown sample from a standard curve. However, it can be done manually by putting the observed Ct value for an unknown sample into the formula: (observed Ct $-$ y intercept)/slope. If the measured PCR efficiency is greater than 100%, then the pipetting of the "knowns" is inaccurate or there is a PCR inhibitor in the standard. If this occurs and is not noticed (by not performing the PCR efficiency calculation), then it can lead to an overestimate of the amount of template in the "unknowns" if it does not have the same PCR inhibitor or pipetting error, when using the standard-curve quantitation method.

few or many samples, or to quantify the number of virus particles (viral load) in a given sample. With this method there is no need for detailed optimization; however, it is best when using an assay that has high PCR efficiency. It is not absolutely necessary to include a control gene, but careful quantitation of the unknowns or controlling for loading is important for accurate results. Adding a control gene can help determine if pipetting errors are significant. A standard curve of a "known sample" for each gene on each plate is essential and ideally spans the range above and below the amount of the unknowns. The most common source of a known sample is a plasmid for the gene of interest, and the standard curve is generated based on a serial dilution of a starting amount, which is quantified with a spectrophotometer, fluorometer, or Pico Green (Molecular Probes, Eugene, OR, USA). Another option, and easier to generate if a plasmid is unavailable, is the use of a synthetic oligonucleotide for the entire amplicon. This is only practical if the amplicon is less than about 100 bp. However, due to the relative purity of the oligonucleotide, it is less susceptible to bias when quantified by a spectrophotometer. Together with the greater precision of measurement of the standard and the possibility of calculating the moles of oligonucleotide (hence, number of copies), it is possible to approximate the number of copies of a template in an unknown sample, although not in terms of absolute copy number. Finally, the last option for a standard curve is to use a cell line with a known copy number or expression level of the gene of interest. The unknown samples can simply be quantified by deriving the value from a standard curve generated with known samples from any of the three sources (Fig. 2).

Oligonucleotide design

The Primer Express oligo design software from Applied Biosystems is probably the most widely used oligonucleotide design program for developing real-time Q-PCR assays. It is capable of designing good assays that will work a high percentage of the time. Primer3, a free program from MIT (see Table 1), can also be used to generate good real-time PCR assays, including designs incorporating an internal hybridization probe. Regardless of the software program used, great care should go into the design of the assay. If using a SYBR Green I approach, the PCR primers must not form an appreciable amount of primer-dimer bands. A melting curve analysis of each product is needed to ensure that the fluorescent signal observed is from the desired PCR product. In mRNA expression assays using a hybridization probe, the probe sequence should span an exon/exon boundary if possible (Fig. 4). Also, screening the genome databases with the amplicon sequence helps ensure that an assay design does not detect pseudogenes. The amplicon for the PCR product should be as small as reasonably possible, 70–150 bp for designs using hybridization probes and less than 300 bp for SYBR Green assays.

Developing Q-RT-PCR assays for mRNA expression level measurements

Developing an ideal assay for Q-RT-PCR includes choosing primers that will result in an amplification product that is specific to mRNA and will not amplify genomic DNA. If using SYBER-Green I, this is more difficult but possible. One of the PCR primers needs to span the intron such that 5 or 6 bases of the 3′ end of one primer hybridizes to one exon of the gene, while the remaining portion hybridizes to the adjacent exon. As illustrated in Figure 4A, this design makes it difficult to amplify contaminating genomic DNA. In a more specific design (Fig. 4B), using a TaqMan probe or molecular beacon, the hybridization probe spans the junction of two adjoining exons, making it very unlikely to detect genomic DNA.

Nucleic acid purification and cDNA synthesis

The purity of the nucleic acid used in Q-PCR is critical to getting reproducible results. The most common sources of nucleic acid for Q-PCR studies are cell culture samples or blood products. Standard commercially available methods and kits are sufficient for producing clean nucleic acid samples. Care is needed if using a phenol/chloroform-based method based on the Chomczynski and Sacchi method [51, as any trace amount of phenol can contribute to reduced reverse-transcription efficiencies. In addition, traces of phenol generate an overestimate of the amount of RNA present in samples that are quantified using a UV spectrophotometer, as phenolic compounds absorb light in the UV range. Other tissue-specific contaminants may contribute to reduced reverse-transcription efficiencies and should be reduced as much as possible.

There are several reports of using RNA extracted from paraffin-embedded tissue for Q-RT-PCR; this can be used, although it will be highly degraded. Nevertheless, the RNA purity is still important for generating good data. In these studies [22,23,52] a considerable effort is still given to purifying the RNA, such as a 3-day incubation with proteinase K to remove as much cross-linked protein as possible [23], so that despite the degradation, the RNA is quite pure. The smaller fragments of randomly degraded RNA are not detrimental to the study due to the very short amplicon size of the assay.

DNase treatment of RNA samples is a very common procedure; however, if the assay design includes a hybridization probe that spans an exon/exon boundary, it can be avoided. If there is no choice but to include a DNase treatment step prior to the cDNA synthesis step, such as for an intron-less gene, currently the best product available is "DNAfree" from Ambion (Austin, TX, USA). It performs the enzymatic degradation of DNA in solution without heat inactivation or further RNA precipitation.

One parameter of Q-RT-PCR that is often overlooked is the reverse-transcription step. Without careful attention to this step it is possible to introduce additional variables, pre-

Table 1. Useful websites and on-line links

Organization	Product or service	Website or URL
Applied Biosystems	Q-PCR equipment and reagents	www.appliedbiosystems.com
Applied Biosystems	User bulletin #2	http://docs.appliedbiosystems.com/search
MIT	Oligo design program	www.genome.wi.mit.edu/cgi-bin/primer/primer3_www.cgi
Santa Lucia Lab	Oligo Tm calculations	http://jsl.chem.wayne.edu/hyther/hytherm1main.html
Stratagene	Q-PCR equipment and reagents	www.stratagene.com
Cephied	Q-PCR equipment	www.cepheid.com or www.smartcycler.com
Roche	Q-PCR equipment and reagents	www.roche.com
Lark Technologies	Custom Q-PCR service	www.lark.com
Q-PCR.com	Q-PCR assay design and training	www.q-pcr.com
Ambion	RNA products	www.ambion.com
Integrated DNA technologies	Oligo's and dual labeled probes	www.idtdna.com
Operon	Oligo's and dual labeled probes	www.operon.com
Biosearch Technologies	Oligo's and dual labeled probes	www.biosearchtech.com
Molecular Probes	Pcio/SYBR Green I dyes	www.probes.com
Q-PCR Listserve*	A helpful Q-PCR discussion group	http://groups.yahoo.com/group/qpcrlistserver/

*Formerly the 7700taqman listserve that existed at Northwestern University.

venting accurate expression analysis. Choice of methods to generate cDNA include random hexamer, oligo-dT, or gene-specific priming using one of several types of reverse transcriptase (RT). The preferred method to generate cDNA is reverse transcription of total RNA with a MMLV-based RT enzyme using random primers. The use of random hexamers will produce the least bias in the resulting cDNA and is amenable to both relative and standard-curve quantitation methods. Further, it is a resource than can be used in later experiments to measure additional genes and still be directly comparable to the expression levels of a previous study when using the relative quantitation method. A potential bias in the production of cDNA from RNA can occur when using only gene-specific primers or oligo-dT. Sequence-dependent interactions, either hairpins in the primer and/or RNA, may contribute to some gene-specific primers having higher RT efficiencies than others. This is not a significant problem when using a standard-curve quantitation method for any given gene, but it can be a significant source of variation when comparing expression levels of different genes, especially when using a relative quantitation method. Also, the use of oligo-dT primers can generate a biased RT product due to RNA secondary structure and length of polyA tail, and it will preclude the use of the 18S ribosomal gene as a control gene with the relative quantitation method. The distance of the amplicon from the polyA or gene-specific RT priming site will contribute to error when using the relative quantitation method, but only when comparing the expression level of one gene to that of another gene.

A two-step RT-PCR approach, in which the reverse-transcription reaction is performed in a separate tube to that of the PCR, is preferred over a one-step RT-PCR reaction, in which both reactions are performed in the same tube. Primarily, the two-step method produces cDNA that can be used in many experiments with several different genes and with less handling of the RNA, which can lead to degradation. The primary advantage to a one-step RT-PCR method is to perform a multiplex reaction in which two separate PCR reactions are being conducted in the same tube using two different-colored probes. This will ensure that both reactions are performed on exactly the same amount of cDNA, but this can also be achieved by a master-mixing strategy in which all components are mixed together with the exception of the primers and probe/beacon, which are then added separately. The second potential advantage to a one-step RT-PCR method is a possible increased sensitivity of the assay, since all the RNA in the RT reaction is also in the same tube for the subsequent PCR step. However, the one-step method has an additional complication of the interaction and influence of one PCR reaction on that of the other reaction when in the same environment. If one gene is expressed at a much higher level than the other, then reagent limitation or pyrophosphate accumulation as a result of the PCR from the gene expressed at higher levels can inhibit the generation of signal from the second PCR reaction. Moreover, the spectral overlap of the two different probes may influence the quantitation of either gene.

Developing Q-PCR assays for DNA copy number measurements

For assays designed to measure DNA copy number or viral particle number, far fewer factors need to be considered for a good assay design than for Q-RT-PCR assays. The amplicon needs to be designed either within an exon or including a portion of the intron for it to be specific to genomic DNA. Both relative- and standard-curve quantitation methods can be used. The standard used for generating the standard curve can be as that of Q-RT-PCR assays, but the choice of endogenous control genes can be different for the relative quantitation method. In addition to using a gene known to be present at two copies, a mixture of primers flanking mic-

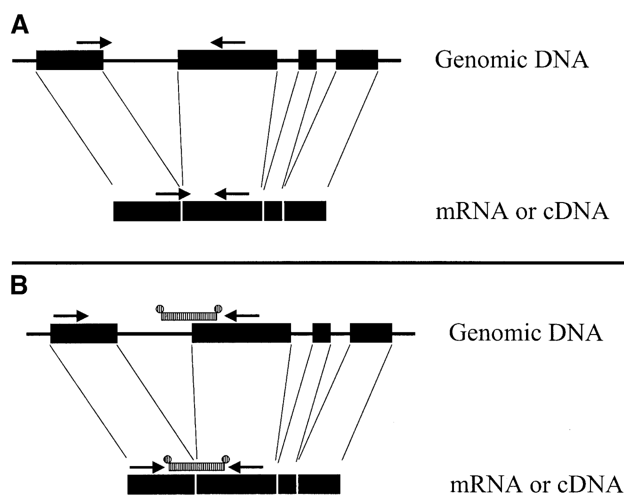

Figure 4. A schematic representation of a typical gene with the preferred position of primers **(A)** or primers and hybridization probe **(B)** for a real-time PCR assay. In the upper panel of each example is the genomic organization depicting the exons (black rectangles) and introns (thin lines); the lower panel depicts what the processed mRNA would look like with respect to exon-exon boundaries once converted to cDNA. In panel **(A)**, the PCR primers (black arrows) are designed such that they span an intron in the genomic DNA, with about 5 or 6 bases of the 3′ end of one primer being complementary to the adjacent exon. A large region of intron prevents the primer from priming a polymerization reaction on genomic DNA template. When drawn in the context of the cDNA, the primers will be able to prime the PCR reaction and will provide specificity for cDNA with little chance of generating PCR product from contaminating genomic DNA. In panel **(B)**, a similar design is depicted with the addition of a dual-labeled probe or molecular beacon (small striped rectangle with small circles representing the fluorescent labels) spanning the exon-exon junction, thereby providing more specificity to a real-time Q-PCR reaction in which a fluorescent signal can only be produced when primed from a cDNA template. This represents the optimal design for a real-time Q-PCR assay as it will simplify the RNA purification process by obviating the need for a DNase I treatment step as well as eliminate the possibility of a mis-priming event generating detectable signal, as might occur when not using a hybridization probe.

rosatellite markers can be used as the “control gene” to reduce the probability that any given locus may be over- or underrepresented in samples with aneuploidy, such as cancer cells [12]. The DNA purity is the primary concern for template preparation of both fixed archival and fresh samples [50]. Ideally, each DNA sample should be tested with a standard curve to determine if there are any PCR inhibitors present, which may prevent accurate quantitation.

Limitations of Q-PCR

With each of the two analytical methods, relative and standard-curve quantitation, there are significant limitations and potential pitfalls. When performing standard-curve quantitation each gene requires a standard curve, which takes up a lot of space on the standard 96-well plate. However, with the 384-well format of the AB7900, this is less of a problem. The need for a plasmid, oligonucleotide, or other source for the standard curve is an extra requirement and can lead to variation, making it difficult to compare data from different plates. Additional plate controls can be used to reduce this variation, but this further reduces the number of samples that can be run per plate. Furthermore, the samples used in generating the standard curve will vary and will make it difficult to compare data generated in different labs unless, for instance, the data are normalized to a standard cell line. When using the standard-curve method, an additional source of error can be introduced at the RT step as different RNA samples may have variable RT efficiencies. However, despite these limitations, the standard-curve method is still a useful method. For a small study it can be very quick to get up and running and it does not require a lot of assay optimization, although PCR efficiency should be calculated and optimized as much as possible. Also, it does not rely on the assumption that an endogenous control gene is unaffected by the experimental conditions.

Relative quantitation is best applied when there are a lot of genes to be tested on many samples. Typically, only one set of wells (usually run in triplicate) is required for each sample tested, maximizing the number of samples per plate. Although relative quantitation relies on the assumption that the endogenous control gene does not vary under the experimental conditions, it is possible to determine if the effect is significant. This can be done by quantifying many test samples of interest with several candidate endogenous control genes. With careful quantitation of the starting template (using RiboGreen I or PicoGreen, Molecular Probes, Eugene, OR, USA), and using a RT protocol that has been proven to be linear, it is possible to determine if any of the potential control genes are consistent enough among samples spanning the experimental conditions to be used as a valid endogenous control. Although this is only an approximation, it should be possible to define those candidate control genes that are significantly affected by the experimental conditions. Control genes that have been successfully used include β-glucuronidase (β-GUS), GAPDH, 18S ribosomal RNA, Histone 3.3a, ubiquitin, and several others.

Future applications of real-time Q-PCR

Now that real-time Q-PCR methods have reached the scientific mainstream it will be exciting to watch the future applications of this technology. Of the most interesting is the combination of real-time PCR with either advanced microdissection techniques [21,53,54] or nucleic acids from paraffin-fixed archival samples [22,23,52,55] or whole-transcript amplification from very small numbers of cells [24]. It will be possible to measure gene expression or DNA copy number in specific cell types that can only be isolated with difficulty and are not very abundant. Other exciting developments for Q-PCR will come in the application of real-time techniques to the analysis of clinical samples to help enable clinicians to stratify and treat patients. The detection and analysis of minimal residual disease [56–58] as well as viral loads will continue

to be important avenues for research. In addition, determining the safety of cell-derived biological compounds [59] or quantification of retrovirus-like particles [60] will be enhanced with real-time Q-PCR. Other applications will help identify potential contaminants during the production of recombinant mAbs for therapeutic use [61]. Combining techniques for sorting fetal cells or DNA from the maternal circulation with Q-PCR will enable early prenatal diagnostics of numerous congenital disorders using minimally invasive procedures [62–65]. Confirmation of expression levels of selected genes from microarray experiments will continue to be conducted using real-time PCR methods [17–19]. With the use of MGB probes, allele-specific expression analysis will be possible as well as the development of highly specific assays that can be performed in the field for use in screening for evidence of biological weapons.

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