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Review

Quantitative Real-time RT-PCR: Application to Carcinogenesis

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Abstract. *Quantitative real-time reverse transcription polymerase chain reaction (RT-PCR) has simplified and enhanced the quantification of gene expression. However, since no agreed standardizations are available, care must be exercised when designing experiments, including the choice of appropriate amplification primers, detection chemistry and the normalization procedure, in order to obtain meaningful results. Coupling quantitative polymerase chain reaction (qPCR) to cell purification from tumor tissue has made it possible to decrease the variability in expression from in vivo heterogeneous cell populations. Sensitive and specific qRT-PCR has advanced the diagnosis, prognosis and prediction response of colorectal cancer to therapy.*

Real-time reverse transcription polymerase chain reaction (RT-PCR) has substantially enhanced the area of gene expression measurement by combining amplification and detection through advances in novel instrumentation, new fluorescent chemistries and advanced bioinformatics. There are two steps to this technique: a) reverse transcription (RT) of mRNA, and b) real-time PCR. RT involves production of cDNA from mRNA. PCR, which was envisaged by Kary Mullis in the mid 1980s as an exponential technique, is based on the exponential amplification of DNA by a thermostable polymerase using a pair of synthetic oligonucleotide primers, each hybridizing to one of the opposite strand of a double-stranded (ds) DNA target (1). The process consists of three steps: a) denaturation step at 95˚C, b) primer annealing to single-stranded (ss)DNA at ~60˚C, and c) primer extension at ~72˚C. This end-point PCR measures DNA accumulation after a fixed number of cycles, allowing quantitative information on the DNA produced at the plateau phase of the reaction. Over the years, several adaptations to this classic PCR have been made, including semi-quantitative PCR, quantitative competitive PCR (2, 3) and, recently, quantitative (q) real-time PCR (4), which employs a homogenous format (*i.e.*, samples are analyzed in a closed-tube). The detection of PCR products required excessive post-PCR manipulations, including running amplified DNA on agarose gels or PAGE and ethidium bromide or other staining, followed by Southern blotting, phosphorimaging or changed coupled device (CCD) visualization for product confirmation, which are time-consuming and employ hazardous chemicals.

The development of a new procedure, in the early 1990s, for analysis of DNA or RNA, based on fluorescent-kinetic RT-PCR, enabled quantification of the PCR products during the exponential phase of the PCR reaction as rapidly as the amplification process itself; thus, requiring no post-PCR manipulations (4, 5). The steps employed during a quantitative RT-PCR are illustrated in Figure 1.

Sample Preparation and RNA Extraction

Studies carried out on tumor cells *in vivo* and *in vitro* have revealed significant heterogeneity in the expression of myriad phenotypic parameters (*e.g.*, difference in karyotype, antigenicity, immunogenicity, biochemical properties, growth, behavior, metastatic capabilities and

Abbreviations: bp, base pair; CCD, charged coupled device; CRC, colorectal cancer; cDNA, copy deoxyribonucleic acid; DABCYL, 4-4'-dimethylaminophenylazol benzoic acid; DPI_3, dihydrocyclo-pyrroloindole tripeptide; DHPLC, denaturing high performance liquid chromatography; ds, double-stranded; FAM, 6-carboxyfluorescein; HEX, hexachloro-6-carboxyfluorescein; JOE, 2,7,-dimethoxy-4,5-dichloro-6-carboxyfluorescein; LCM, laser capture microdissection; mRNA, messenger ribonucleic acid; PAGE, polyacrylamide gel electrophoresis; RT-PCR, reverse transcription polymerase chain reaction; ss, single-stranded; TAMRA, 6-carboxy-N,N,N',N'-tetramethylrhodamine; TET, tetrachloro-6-carboxyfluorescein.

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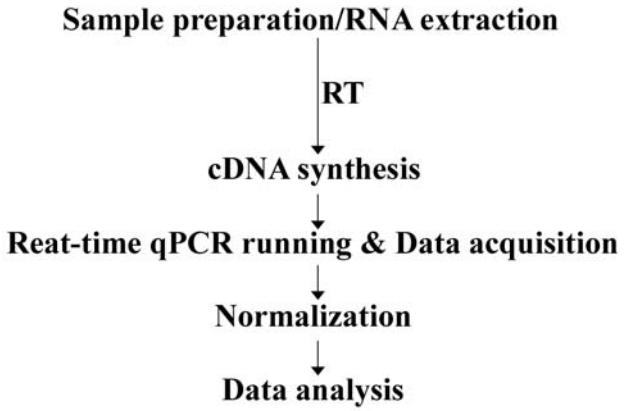

Figure 1. *Steps performed when studying mRNA expression using real-time PCR.*

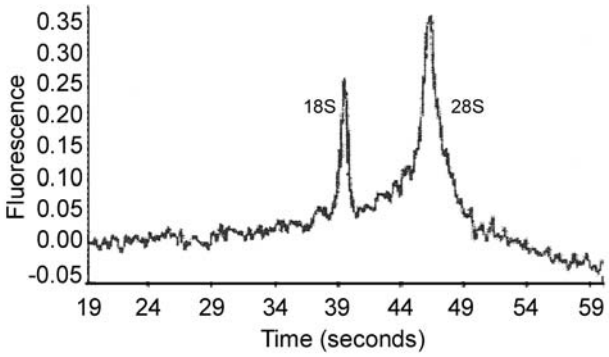

Figure 2. *Electrophoretogram of non-degraded total RNA colonic sample as judged by the presence of 18S and 28S rRNA bands.*

cellular susceptibilities to chemotherapeutic agents) in both primary and non-metastatic lesions, including stroma and surrounding non-cancer tissue (6). Significant differences have been detected in gene expression profiles between microdissected and bulk tissue samples (7). Hence, it is essential to use an enrichment method to separate tumor cells from benign and inflammatory cells before isolating RNA to diminish the undesirable background expression levels (8).

The introduction of laser capture microdissection (LCM) in the mid 1990s allowed for the isolation of pure populations of intact tumorigenic cells from specific microscopically-defined regions of excised frozen tissue by briefly pulsing an infrared laser to target cells, selectively adhering those cells to a thermoplastic polymer film and removing them undamaged to a sterile plastic vial for subsequent extraction of high quality RNA (9). LCM has also been applied to archival formalin-fixed and stained tissue (10). Although the RNA extracted from such preparations is partially degraded, it is possible to carry out an accurate and reproducible qRT-PCR because amplicons as small as 60 bp can be employed (11). The isolation of RNA from a small number of cells by traditional methods is inefficient for many samples. Therefore, column-based extraction kits have been developed by various manufacturers, allowing for rapid isolation. To obtain reliable quantitative results, the extracted RNA must conform to certain criteria: a) it must be undegraded, b) be free of genomic DNA, particularly if the target is an intronless gene, c) be free of inhibitors of the RT step and d) be free of nucleases for extended storage (12). A capillary electrophoresis instrument (*e.g.*, Agilent 2100 Bioanalyzer, Palo Alto, CA, USA) in conjunction with the RNA Lab Chip are convenient for assessing the quality and quantity of a large number of RNA samples (13) (see Figure 2).

A study carried out on quantifying RNA by various methods ($A_{268/280}$, Nanodrop, Ribogreen and Agilent) found significant differences in the quantification results, suggesting that it is inaccurate to compare data between preparations using different quantification methods (14).

Reverse Transcription

The step of converting RNA into a cDNA template contributes to the variability in qRT-PCR experiments because: a) the dynamic state of the cell causes inherent variation in the RNA extracted from various samples, b) purified RNA is inherently unstable and may be of variable quality, c) the efficiency of the RNA-to-cDNA conversion depends on template abundance, being significantly lower when target templates are rare, and negatively affected by background nucleic acid in the RT reaction (15), and d) it depends on the reverse transcriptase used. A study comparing several transcriptases showed the RT yield of the multistrand to vary by more than 100-fold, and variations were gene-dependent. Avian myeloblastosis virus (AMV; Promega, Madison, WI, USA) gave the lowest yield (0.4%) for 106 RNA molecules, while SuperScript III RNase H– (Invitrogen, Carlsbad, CA, USA) was overall the most efficient reverse transcriptase, giving the highest yield (90%) for 10^4 RNA molecules, with a mean yield of 83%. Moloney murine leukemia virus (MMLV, Promega) and RNase H^-MMLV (MMLVH; Promega) gave mean yields of 44% and 40%, respectively, whereas the mean yields of other reverse transcriptases employed (*e.g.*, Omniscript, Qiagen, Valencia, CA, USA; Improm-II, Promega; cAMV, Invitrogen; and ThermoScript RNase H-, Invitrogen) were <25% (16).

Copy deoxyribonucleic acid (cDNA) priming can be accomplished using random primers, oligo-dT, or target-specific primers. Each of these methods produces a

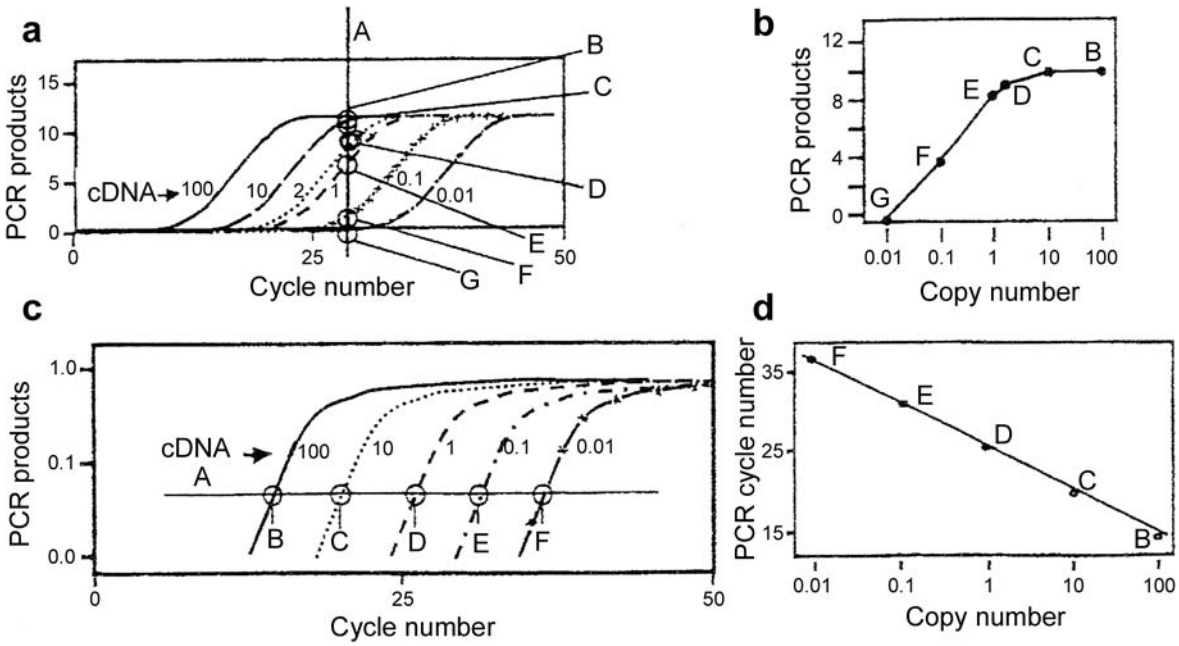

Figure 3. *Illustration of difference in performance between conventional and real-time PCR.*

different cDNA yield and specificity. Random hexamers, employed in ~30% of reactions, prime RT at multiple points along the transcript; hence, producing more than one cDNA transcript per original target. This method is non-specific, yields the most cDNA, generates the least bias in the resulting cDNA, and is useful for transcripts with a significantly secondary structure (12). However, the majority of synthesized cDNA is ribosomal (r) RNA. This could lead to ineffective priming if the target amplified is present at low levels; thus, its amplification may not be quantitative. Random hexamers were shown to overestimate mRNA copy numbers by up to 19-fold compared with a 22-mer sequence-specific primer (17).

Oligo-dT, used in ~40% of assays, is more specific to mRNA than random priming as it does not transcribe rRNA. It attempts to generate transcripts from mRNA with a significant secondary structure or if the primer/probe binding site is at the extreme 5'-end of a long mRNA, and will not prime RNAs that lack a polyA tail. However, since oligo-dT priming requires a full-length quality RNA, it is not a good choice for transcribing RNA that is likely to be fragmented (such as that obtained from archival material) (12). Approximately 10% of qRT-PCR assays use a combination of oligo-dT and random hexamers. While this approach may be acceptable for qualitative assays, it could exacerbate the problems inherent with the individual methods, and should only be employed when accurate quantifications are not necessary (13).

Target-specific primers, used in ~25% of assays, synthesize the most specific cDNAs. A reaction primed by target-specific primers is linear over a wider range than a similar reaction primed by random hexamers and produces superior results compared to random priming. Its main disadvantage is the requirement for separate priming reactions for each target, which makes it impossible to return to the same preparation to amplify other targets later on. It is also wasteful if only limited amounts of RNA are available (12, 13). Target abundance may also influence the choice of the primer for the RT step. For example, RT using specific primers may be appropriate for an abundant target, while random priming may be preferable if the target is present in low copy numbers (18). Primers specific for RT can also be used for a one-step PCR amplification.

Another factor that impacts RT is the "Monte Carlo" effect (an inherent limitation of PCR amplification from small amounts of any complex template due to differences in the amplification efficiency between individual templates in an amplifying cDNA population). The Monte Carlo effect is dependent upon the template concentration, with less abundant templates unlikely to be truly reflected in the amplified product. The cDNAs of less abundance are more likely to experience the Monte Carlo effect since their probability of primer annealing is lower. A proposed solution to this problem is to use mRNA instead of total RNA preparations. However, mRNA preparations involve additional steps, which may lead to sample loss, making it

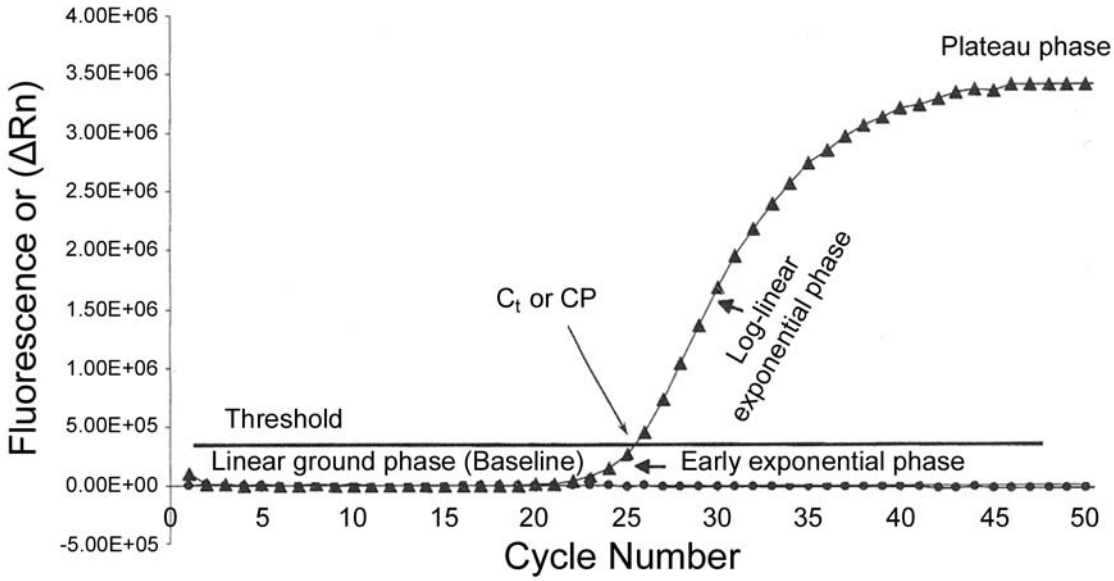

Figure 4. *Amplification curve of real-time RT-PCR of log fluorescence (or ΔRn) versus cycle number showing the 4 phases of the PCR reaction. The instrument algorithm calculates ΔRn using the equation* $\Delta Rn = Rn^{+} - Rn^{-}$, *where* Rn^{+} *is the fluorescence emission of the product at each time-point estimated by dividing the fluorescence emission of the reporter dye by the fluorescence emission of a passive reference dye such as ROX (usually incorporated in the PCR master mix to control for differences in master mix volume), and Rn- is the fluorescence emission at the baseline.*

more difficult to assess the quality of the final product, or worse mRNA fragmentation resulting from sample contamination (12).

Principles of Real-time PCR

Traditional methods for quantification of mRNA, such as Northern blotting, nuclease protection assays and *in situ* hybridization, have been falling out of favor due to their inferior sensitivity and accuracy compared to qRT-PCR (8). Real-time PCR refers to the analysis of product kinetics as they accumulate. By plotting the increase in fluorescence (or PCR product) *versus* the cycle number, the system produces amplification plots that provide a large dynamic range of target molecule determination ($>10^7$-fold) as compared to traditional PCR; a feature allowing for a higher sample throughput, decreased labor and cost, and increased fluorescence (5). In theory, the production of PCR products should proceed exponentially. However, in practice, it reaches a plateau after roughly 30 to 40 cycles, as certain reaction components become limiting. In conventional PCR, products of the reaction are measured at a single point in the reaction profile, as indicated by the vertical line A in Figure 3a. Plotting the concentration of products present at point A *versus* copy number present in the original sample shows that proportionality between the copy number and PCR products occurs over a limited dynamic range (between G and E in Figure 3b), leading to loss of precision in quantification. On the other hand, it has been shown empirically that product concentration in the RT-PCR reaction is proportional to the PCR cycle number during the exponential phase of PCR. Therefore, if the number of cycles it takes for a sample to reach the same point in its exponential growth curve is known (horizontal line A in Figure 3c), the precise product concentration or percentage can be determined (Figure 3d).

Figure 4 illustrates a typical amplification plot of a real-time RT-PCR reaction showing: a) baseline (or linear ground phase), which usually encompasses the first 15-20 cycles; b) early exponential phase where fluorescence reaches a threshold (usually 10 times the standard deviation of the base line). The chosen cycle at which this occurs is known as the threshold cycle (Ct) or the crossing point (CP) depending on the thermocycler used. This value is representative of the starting copy number in the original template and is used to calculate experimental results (5). PCR reaches its optimal amplification potential during the log-linear phase, with the PCR product doubling after every cycle in ideal reaction conditions (8); d) the plateau stage is reached when the reaction components become limited due to depletion of the PCR components, decline of polymerase activity and competition with PCR products, so that the fluorescence intensity is no longer useful for data calculation (19-21).

Instrumentation and Automation

Today, over 63 companies provide PCR-related products and/or services. In March, 2005, a series of core process patents covering the PCR technique expired in the USA; they will expire worldwide by next year. The first available instrument, the 7700 SDS™ from Applied Biosystems (Foster City, CA, USA) took up almost an entire bench space, used an expensive high-intensity laser light source, and needed to be placed in an air-conditioned laboratory. It was not really a real-time apparatus since data could only be viewed after the end of the run. The newer instruments are much smaller in size and the laser has been replaced by less expensive tungsten-halogen or light-emitting diode lamps. Each instrument has its own advantages and disadvantages, and the choice of a particular thermal cycler depends on its intended use (22). There is currently a need to increase the throughput and speed by adapting thermal cyclers to handle 96- and 384-well plate formats, and to employ automatic liquid robotic handlers to increase precision. Another way to increase throughput is through multichannel detection, where up to six different fluorescent dyes can be used in a single reaction. Several manufacturers are also increasing the velocity of the reaction to achieve greater speed. For example, Strategen, La Jolla, CA, USA, now employs a heat-tolerant DNA polymerase (FullVelocity™) engineered from organisms of the species *Archea*, which are found in extreme environments (22). Another aspect of PCR speed is the physical limitation of the current cyclers. In order to have good, accurate, sensitive PCR results, it is important for the instrument to move as quickly as possible between the temperature steps (8, 23). To address this issue, Roche's LightCycler™ (Indianapolis, IN, USA) uses pressurized heated and cooled air, instead of blocks that changes temperature slowly, to maximize heat transfer to samples, which are contained in glass capillaries, giving results in 20 minutes. Recently, however, Roche has solved the problem of rapid heat transfer to blocks, and released its new LightCycler 480™ in a 96- or 384-well format. The future promises faster and miniature quantitative PCR devices, with software designed to give instantaneous results (22).

One- or Two-step PCR Reaction?

In designing an RT-PCR for a specific application, one must first choose a one- or two-step reaction format. One-step allows both cDNA synthesis and PCR amplification to be performed in a single tube, with either one enzyme or an enzyme blend. This minimizes the reaction time and the chance of sample contamination, and is especially useful for experiments that require maximum amplification specificity, although it has been reported to be less sensitive than the two-step protocol (24). The two-step format, on the other hand, allows RT and amplification to be performed separately under optimal conditions. This method is particularly attractive for experiments that require the same RT product to be used for analysis of multiple transcripts, although it increases the opportunity for DNA contamination due to increased sample handling. Whether using a one- or two-step process, cDNA synthesis greatly affects the overall RCR results. Both reverse transcriptase and dithiothreitol (DTT) are PCR inhibitors that may affect reaction kinetics in a one-step process, or when carried over in a two-step reaction. Additionally, samples from complex biological sources often have other PCR inhibitors that may be carried over during sample preparation. Inhibitor carry-over can be avoided using a cDNA precipitation protocol, while DTT could be entirely omitted from the reaction (20).

Chemistries of Detection

There are currently several techniques for detecting purified products with nearly the same sensitivity, but with different specificities: a) non-specific detection using intercalating dyes, and b) specific detection using various probes and hairpin structures.

DNA binding dyes. This technique was first described by Higuchi *et al.* (4), who monitored the increase in ethidium bromide fluorescence using a CCD camera. More recently, SYBR Green I dye, being less toxic and more specific than ethidium bromide, and incorporating into the minor groves of dsDNA has been used (Figure 5a). During the PCR reaction, the amount of ds target increases, paralleled by an increase in the dye's incorporation and fluorescent emission. The advantages of using this method are low cost and reduced labor. However, because the dye does not bind in a sequence-specific manner, the assay is prone to false-positive results (25), unless a melting curve analysis is performed to discriminate between specific and non-specific PCR products that melt at lower temperatures (26) (Figure 6). Another drawback of this method is that multiple dye molecules bind to an amplified target; thus, the signal generated depends on the mass of dsDNA produced (*i.e.*, amplification of a longer product generates more signal than a shorter one) (19).

Hydrolysis or TaqMan probes. Real-time PCR was first described using these probes (5). The TaqMan technology (also known as the 5'-nuclease assay) utilizes the 5'-nuclease activity of the DNA polymerase to hydrolyze a hybridization probe bound to its target amplicon. The probe emits a fluorescent signal upon cleavage based on the principle of fluorescence resonance energy transfer (FRET). The probe is non-extendable at its 3' end and is dual-labelled, with a reporter fluorochrome (such as FAM, HEX, JOE or TET),

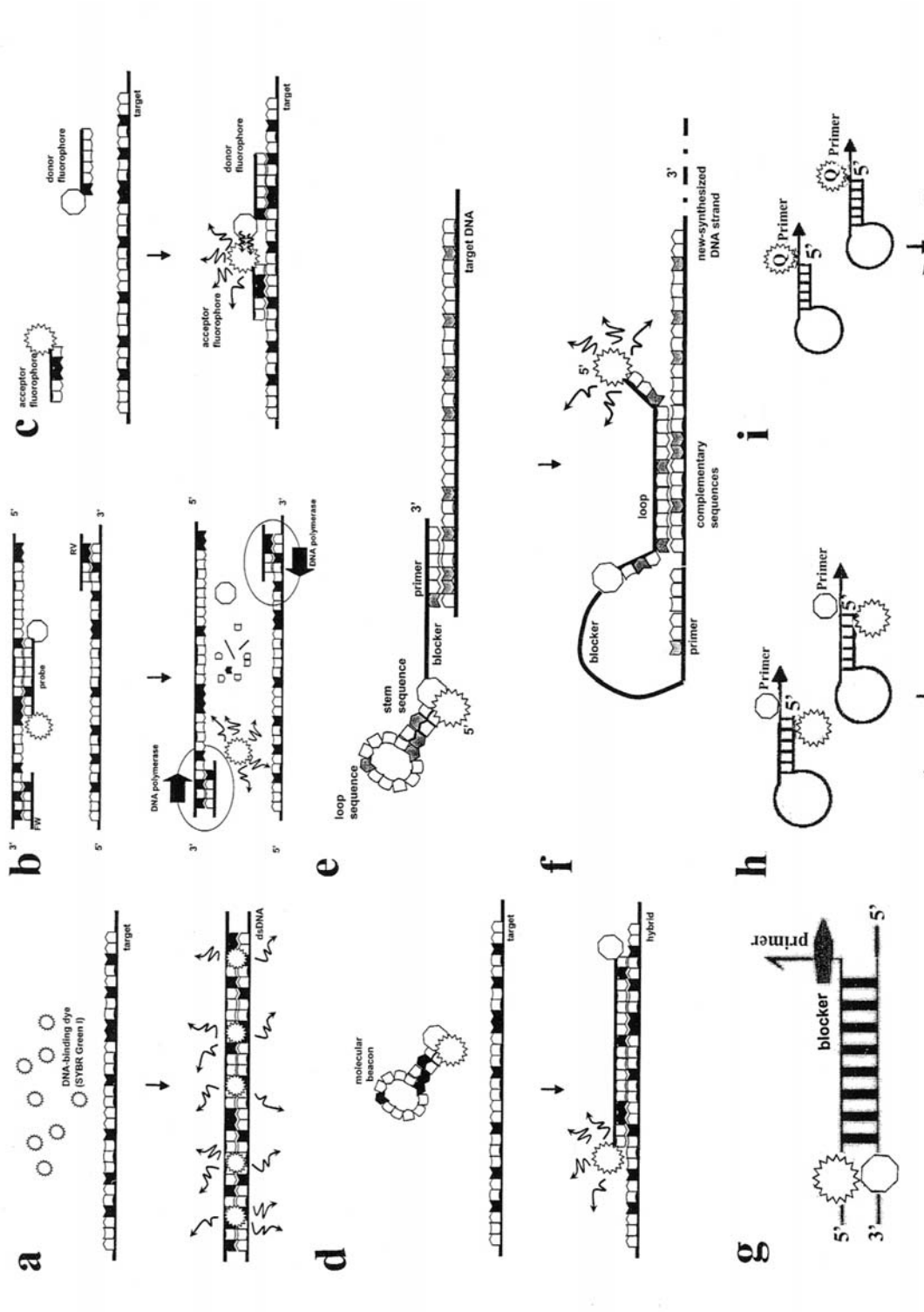

Figure 5. *Detection chemistries. (a) SYBR Green I incorporates into minor groves of dsDNA resulting in fluorescence emission. (b) Hydrolysis TaqMan probe. Forward primer (FW), reverse primer (RV), fluorophore (star), quencher (hexagon). (c) Hybridization probe. When the acceptor and donor fluorophore anneal closely to the target sequence, the donor excites the receptor through FRET and fluorescence ensues. (d) Molecular beacons. When in hairpin-shaped unbound form, the fluorophore and quencher are close resulting in no fluorescence emission, but when the probe anneals to a complementary target sequence, the quencher and fluorophore are apart and fluorescence emission occurs. (e) Unbound quenched scorpion. (f) Bound scorpion. Fluorescent emission occurs because the fluorophore and quencher are apart. (g) Scorpion with the fluorophore and quencher separated into different oligonucleotides. (h) Sunrise primers. (i) LUC primers. Quenched fluorophore (Q-star). (Modified from references 11, 20, 21 and 40).*

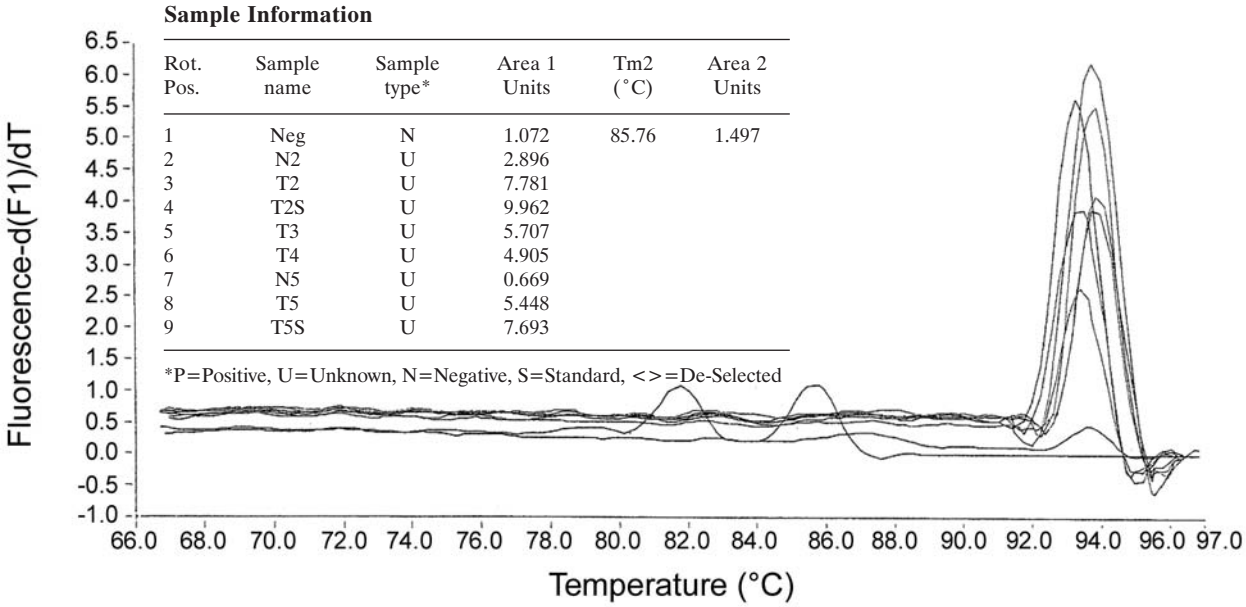

Figure 6. *Melting curve analysis of a colon-specific gene (PYPAF5) using the LightCycler™.*

and a quencher fluorochrome (such as DABCYL or TAMRA). The probe is designed to anneal to the target sequence internally of the primer during the annealing and extension phase of the PCR reaction. In its free, intact form it emits no fluorescence (no FRET) because the fluorescence emission of the reporter dye is adsorbed by the quenching dye. However, upon annealing of the probe to one of the target strands, the probe will become degraded by the 5' → 3' exonuclease activity of the Taq polymerase, resulting in an increase of reporter fluorescent emission, for example, from FAM at 518 nm. This process occurs in every amplification cycle and does not interfere with the exponential amplification of the PCR product. Increased fluorescence in measured in every cycle and is directly correlated with the amount of amplified PCR product (5) (Figure 5b). Minor grove binders (MGBs) such as DPI3 may be added to these probes to increase their T_m and allow the use of a shorter probe, which are not only less expensive, but also have a reduced background fluorescence and a larger dynamic range due to the increased efficiency of reporter quenching (27).

Hybridization probes. As opposed to the above probes, these probes also use FRET to increase the fluorescence intensity. Two sequence-specific probes, also known as HybProbes, are used (27). In the classic probes, each one has a single fluorescent donor fluorophore at its 3' end (*e.g.*, fluorescein, FAM) and an acceptor fluorophore (*e.g.*, Cy5, LC Red640, LC Red705 or ROX) at its 5' end. The sequence of these two probes are designed to anneal to the target sequence in very close proximity (within 1-5 nucleotides) in a head-to-tail arrangement to each other, resulting in emission of light from the donor fluorochrome, which excites the acceptor fluorochrome (FRET) allowing it to dissipate energy at a different wavelength (Figure 5c). The amount of fluorescence emitted can be measured during the PCR annealing phase and is directly proportional to the amount of target DNA (28).

A variant of these probes uses a fluorescently labelled primer/probe combination in which a 5'-labelled hybridization probe is designed to anneal to the PCR strand, in close proximity to one of the PCR primers, which has a fluorophore at its 3' end. The fluorescently-labelled primer is positioned near the probe (within 5 bp) to allow FRET with the complementary probe (29).

Since these probes do not hydrolyze, the fluorescence is reversible, allowing the generation of melting curves (19). Since a single melting curve can distinguish up to four different T_ms, up to six different probes could be multiplexed in a single reaction vessel (20).

Hairpin probes. Several probes belonging to this category are detailed below:

Molecular beacons: Are the simplest hairpin probes consisting of a sequence-specific loop region flanked by two inverted repeats (30). The fluorophore and quencher are in close proximity when the probe is in its free, unbound state. When the probe anneals to the complementary target sequence, its conformation changes, separating the quencher and fluorophore, resulting in fluorescence emission (Figure 5d). These probes can distinguish targets that differ by only

a single nucleotide, and they are much more specific than TaqMan probes of equivalent length because the probe-target complex must be thermodynamically more stable than the hairpin structure (31).

Scorpions: They are single-stranded (ss) dual-labelled fluorescent primer/probes that contain a 5' end fluorophore, and an internal quencher dye directly linked to the 5' end of a PCR primer *via* a PCR blocker to prevent the Taq DNA polymerase from amplifying the stem-loop sequence. This configuration brings the fluorophore in close proximity to the quencher leading to no fluorescence (Figure 5e). When the primer/probe and the target anneals, the hairpin opens and the fluorophore and quencher separate resulting in increased fluorescence emission (Figure 5f). Scorpions differ from TaqMan, hydrolysis probes and molecular beacons as their structure promotes a unimolecular probing mechanism, which results in a stronger fluorescent signal, especially under fast cycling conditions (32). Another advantage scorpions have over TaqMan probes is that the PCR reaction is carried out at the optimal temperature for the polymerase, rather than at the reduced temperature required for the 5'-nuclease assay to displace and cleave the probe (11). However, scorpions are not easy to design. Moreover, this method requires running agarose gels to ensure an amplified PCR product because PCR priming and probe binding are not independent (33).

Scorpions have been improved by the creation of a duplex format, where the reporter dye/ probe and quencher fragment are located on separate complementary molecules, thereby yielding greater signal intensity because the reporter and quencher can separate completely (Figure 5g) (34). The modified scorpion is easer to design and synthesize as there is no hairpin structure (11).

Sunrise primers™: These primers, manufactured by Oncor (Gaithersburgh, MD, USA), are similar to scorpions as they combine both PCR primer and detection mechanisms in the same molecule (Figure 5h). These probes consist of a dual-labelled (reporter and quencher fluorophores) hairpin loop on the 5' end, with the 3' end acting as the PCR primer. When unbound, the hairpin is intact, causing reporter quenching *via* FRET. Upon integration into the newly-formed PCR product, the reporter and quencher are held far enough apart to allow reporter emission (35). This method requires running agarose gels to ensure the formation of a true amplified PCR product.

Light upon extension (LUX™) fluorogenic primers: LUX primers (Invitrogen) are self-quenched single-fluorophore labelled primers, structurally identical to Sunrise primers (Figure 5i), but instead of using a quencher fluorophore, the secondary structure at the 3' end reduces the initial fluorescence to a minimum (36). LUX relies on only two oligonucleotides for specificity. These processes are much less expensive than dual-labelled probes because they do not require a quencher intercalating dye that uses a melting curve to distinguish the true from false amplification product, as no such detection exists for LUX primers. Therefore, agarose gels need to be run to ensure the presence of a single PCR product.

The major disadvantages of specific probes are: a) because of their specificity, artifacts that interfere with amplification efficiency cannot be detected. Therefore, intercalating non-specific dyes should be used first to optimize the primers and reaction conditions prior to any quantification assays to ensure the absence of amplification artifacts (12), and b) the high cost associated with specific chemistries due to the post-synthesis high performance liquid chromatography (HPLC) and/or polyacrylamide gel electrophoresis (PAGE) purification steps which are needed because of limitations in traditional synthetic chemistries which necessitate that each target requires its own specific probe. This becomes quite expensive when quantifying multiple targets (37).

Probe purity. The recent availability of 3'quencher controlled pore glass synthesis support columns that allowed 3' quencher incorporation has presented the possibility that probes – when carefully synthesized – may be used without extensive post-synthetic purification, which would substantially reduce cost. The Nucleic Acid Research Group of the Association of Biomoleculer Resource facilities (Santa Fe, NM, USA) monitored the ability of several DNA synthesis laboratories to synthesize dual-labelled fluorescence probes suitable for qRT-PCR without the need for post-synthesis purification by asking 18 member laboratories to synthesize 35 dual-labelled human β-actin probes and submit them for quality and functional analysis (37). A new variety of a non-fluorescent quencher [the Black Hole Quencher 1 (BHQ-1™; Bioresearch Technologies, Novato, CA, USA)] was compared with TAMRA™ (Glen Research, Sterling, VA, USA). The BHQ-1 quencher has no intrinsic background fluorescence and requires no changes to standard oligonucleotide synthesis procedures, making it possible to complete the synthesis and deprotection of a dual FRET probe on a standard automated DNA synthesizer. In contrast, a non-standard mild deprotection protocol was required for TAMRA-quenched probes. DHPLC (WAVE) analysis demonstrated the ability to resolve full-length probes based on hydrophobicity. Moreover, WAVE data showed that a well synthesized crude probe had a profile similar to that of a purified probe. Additionally, the fluorescence traces demonstrated the problem of TAMRA probes being contaminated with undesirable background fluorescence, unless the probes were extensively purified (Figure 7). Non-purified BHQ-1 probes tended to be of higher purity than non-purified TAMRA

probes and produced higher fluorescence. Several of the non-purified BHQ-1-quenched probes approached the purity of the HPLC-purified probes. However, for the TAMRA-quenched probes, none of the crude probes was more than 75% pure. Probes that were at least 20% pure had the same efficiency as those of near 100% purity, but the sensitivity of the assay was reduced as the level of purity decreased. The cost of a commercially prepared dual-labelled, purified probe at the 200 nmol scale ranges from $200 to $400, whereas the cost of preparing a similar scale probe without post-synthesis purification (Bioresearch Technologies) averages $50-70. If this wise strategy were adopted, it would induce qPCR users to concentrate on perfecting the design of assay primers instead on concentrating on probe quality (37).

Quantification

Quantification is based on the inherent property of a PCR reaction that the more input DNA copies one starts with, the fewer cycles of PCR amplification are needed to make a specific number of an amplification product, as this amplified product correlates linearly with the amount of fluorescent emission (8, 20, 21, 23). Quantification depends on the kind of instrument used, but there are two basic methods: absolute and relative.

Absolute quantification. This quantification can be carried out by spiking the sample with a known amount of an internal control (or absolute standard) that shares the same primer binding sites to target sequence, but contains small amplicon differences (*e.g.*, deletions, insertions or mutations made only in *in vitro* transcribed RNA) but, because of its labor-intensive nature, this method is not widely used. More often, cDNA plasmids standards, constructed by cloning a cDNA fragment into a suitable plasmid vector, are utilized instead, which results in a relative quantification because variations in the efficiency of the RT step are not controlled (21, 23).

Absolute quantification uses serially-diluted standards of known concentrations of the control to generate a standard curve. The standard curve produces a linear relationship between C_t (or CP) and the initial amounts of total RNA or cDNA, allowing the determination of the concentration of unknowns based on their C_t value. This method assumes that standards (*e.g.*, dsDNA, ssDNA or any cDNA or cRNA expressing the target sequence) and samples amplify with similar efficiencies (38). Moreover, the concentrations of the serial dilutions of standards must encompass the levels in the experimental samples, and stay within the range of accurately detectable and quantifiable concentrations specific for both the PCR apparatus and assay parameters (20). DNA standards have a larger quantification range and greater sensitivity, reproducibility and stability than RNA standards; however, a DNA standard cannot be used for a one-step qRT-PCR due to the absence of a control for the RT efficiency (39, 40).

The concentrations of the samples can be measured spectrophotometrically at 260 nm and converted to the number of copies using the convention (41):

$$\frac{6\text{x}10^{23}\ \text{(copies/mol)} \times \text{concentration (g/µl)}}{\text{MW (g/mol)*}} = \text{amount (copies/µl)},$$

where

*Molecular Weight (MW) = (number of bp, or length of primers used in bp) X [average MW of dsDNA (660 daltons/bp); using 1 mol = $6\text{x}10^{23}$ (copies).

The slope of the standard curve is a measure of the efficiency of the PCR reaction. For serial 10-fold dilutions, it should ideally be –3.3. In practice, however, slopes between –3.0 and 3.6 are encountered. Moreover, the sensitivity of the PCR reaction is reflected in the standard curve by the point at which the standard curve crosses the Y axis (Y intercept); the lower the Ct value at this point, the higher the sensitivity of the PCR reaction (Figure 8B). By plotting the Ct value of an unknown sample on the standard curve, the amount of input target sequence in the sample can be determined automatically by the software program of the PCR instrument (40).

Relative quantification methods. Several methods and arithmetic models are available to accurately estimate the mean normalized gene expression from relative quantification data. A brief survey is given below:

Standard curve method: This method estimates sample gene expression based on an external standard (or a reference sample, calibrator), designated as 1-fold, with all experimentally-derived quantities reported as the n-fold difference relative to the calibrator. Only the relative dilution factors of the standards used for quantification are needed (8, 42, 43). By plotting the C_t value of an unknown sample on the standard curve, the amount of an unknown sample can be determined. This method is often used when the amplification efficiencies of the reference and target genes are unequal. It is also the simplest quantification method because it does not require the preparation of exogenous standards or quantification of calibrator samples, and is based on simple principles. However, because it does not incorporate an endogenous control housekeeping gene, the results need to be normalized (20).

The comparative C_t, or (the $\Delta\Delta C_t$) method: This method (also known as the comparative cross point method) uses arithmetic formulae to calculate the relative expression levels of a target

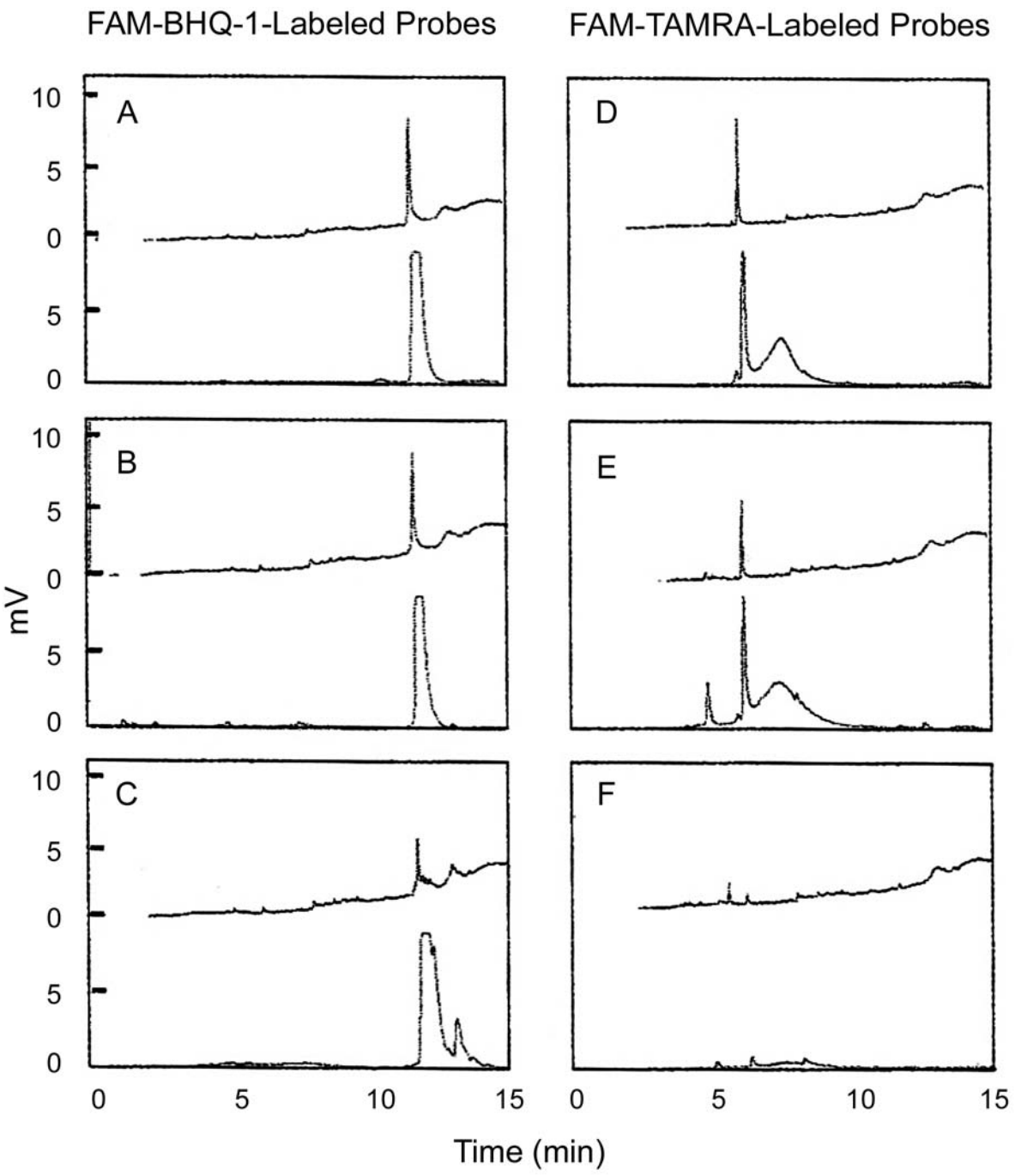

Figure 7. *Resolution of probes by hydrophobicity utilizing DHPLC. The top and bottom curves of each panel are the UV and fluorescent elution traces in millivolts (mV), respectively. (A-C), BHQ-1-quenched probes eluting at 12.5 min. (D-F) TAMRA-quenched probes eluting at 6.7 min. (From reference 37, with permission).*

compared to a control calibrator, then the value of the unknown target is normalized to an endogenous housekeeping gene. The amount of target is measured by the equation: $2^{-\Delta\Delta Ct}$, where $\Delta\Delta Ct = \Delta C_{t,\ sample} - \Delta C_{t,calibrator}$, and ΔC_t is the C_t of the target gene subtracted by the C_t of the housekeeping gene. The equation thus represents the normalized expression of the target gene in the unknown sample, relative to the normalized expression of the calibrator

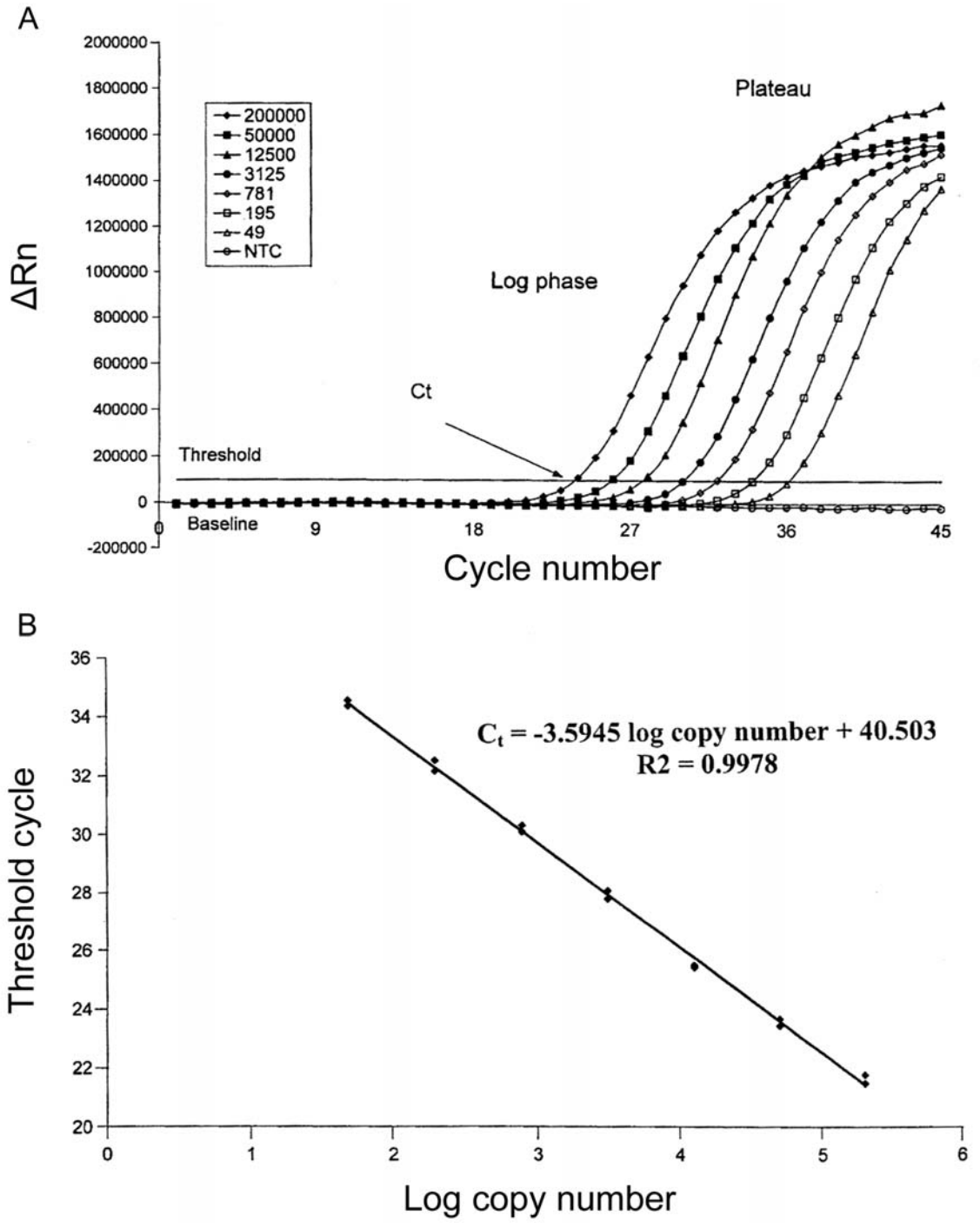

Figure 8. *Amplification plots of IL-4 plasmid cDNA. (A) Five-fold serial dilutions of IL-4 plasmid cDNA were amplified by RT-PCR using the ABI Prism 7700 SDS™. The software constructs amplification plots where ΔRn is plotted against cycle number. (B) Standard curve of IL-4. C_t values are plotted against the log concentration of input cDNA copy number. The slope of the curve, which is a mean of measuring amplification efficiency, is –3.5945. (From reference 40, with permission).*

sample (44). The machine software algorithm identifies the first turning point (C_t or CP) using a second derivative maximum method (8, 27). The $\Delta\Delta C_t$ method can only be used if the PCR amplification efficiency of the target and housekeeping genes is equal. If not, a new set of primer/probe combinations has to be designed.

The amplification efficiency of the reaction is an important consideration when performing relative quantification. In an ideal situation, the amplification efficiency equals 1, meaning that the PCR products double each cycle during the exponential phase of the reaction. However, in reality, the amplification efficiencies are not ideal, and calculations made without an appropriate correction factor often overestimate the starting concentration (42).

Traditionally, the amplification efficiency of a reaction is calculated from a standard curve using:
Efficiency = $[10^{(-1/\text{slope})}] - 1$.
The amplification efficiency of the reaction declines from being relatively stable in the early exponential phase to zero in the plateau phase. Calculations of the amplification efficiency using a standard curve do not usually reflect this changing efficiency and may overestimate it (42). However, since PCR results are based on C_t values, which are determined early on in the exponential phase of the reaction, these differences in amplification efficiency generate only minor differences in the C_t value but, after ~30 cycles, a 5% difference in amplification efficiency may result in a 2-fold difference in the PCR product concentration. The amplification efficiency calculated from raw data appears to be more accurate than when derived from a standard curve (43).

Pfaffl model: This model combines gene quantification and normalization into a single calculation, and incorporates the amplification efficiencies of the target and reference (normalization) genes to correct for differences between the two assays (45). The relative expression software tool [REST©], which runs in Microsoft® Excel, automates the data analysis in this model. REST uses the Pairwise Fixed Reallocation Randomization Test© to calculate the significance of the result, and will also check the suitability of the reference gene for normalization (46).

Q-gene, or Muller et al. method: This is a comprehensive Microsoft Excel-based software application that aids the entire process of qRT-PCR, from experimental planning and set-up to data analysis, statistics and graphical presentation (47). The method calculates the mean normalized gene expression with standard error using two different mathematical models that correct for amplification efficiencies. The calculated expression values are then compared with two matched groups to determine the expression of a sample relative to a calibrator. This method is particularly convenient when running complex qPCR because of its data management capabilities.

Gentle et al. method: In this method, linear regression analysis of the raw log fluorescent exponential PCR data is used to calculate the amplification efficiency of the samples, without using standard curves. By graphing the control and experimental samples together, the vertical distance between the control and experimental lines is the log of the fold difference between the two, and the slopes of the lines represent the log of their amplification efficiencies. This method uses an Excel spread sheet to enhance the data processing (48).

Liu and Saint method: The authors developed a sigmoid mathematical model to quantitate and normalize mRNA gene expression. As in Gentel *et al.* (48) above, this model calculates the amplification efficiencies from the actual slope of the amplification plot, rather than from a standard curve. This method was reported to be more accurate than the $\Delta\Delta C_t$ (44) method regarding the varying amplification efficiencies through the PCR cycle, since the user defines which PCR cycles exhibit exponential amplification and uses them for calculations (49).

Amplification plot or DART-PCR method: This method uses a simple algorithm to individually calculate the amplification efficiency of every sample in the qPCR assay, and then uses raw data for quantification of the expression *via* a Microsoft Excel workbook titled "Data Analysis for Real-Time PCR (DART-PCR)" (50).

Pros and cons of quantification approaches. A drawback to amplifying standard and target genes in the relative quantitative approach is that amplification of the abundant control may overwhelm the signal of the mRNA target of interest; thus, extra care must be taken to ensure that the expression levels of the control and the target match closely. By modulating the amplification efficiency of the control in multiplex PCR without affecting that of the other templates in the reaction, Ambion (Austin, TX, USA) has developed a competimer technology to solve this problem. Competimers are oligonucleotide primers with the same sequence as the control primer, but they are modified at the 3' end so that they cannot be extended by DNA polymerase, thereby limiting the amplification of the control gene. Using this technology, both Quantum® 18S RNA and β-actin Internal Standards are available, with 18S RNA appearing less variant than that of β-actin or glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (51). TaKaRa offers a human β-actin Competitive PCR set, distributed by the Pan Vera Corporation (Madison, WI, USA) to normalize RNA concentrations from different samples. The concentration of the competitor that generates a template competitor ratio of 1:1 may also be used to establish the relative amounts of initial target in the sample (51).

The notion that relative qPCR methods are inferior to absolute ones has been challenged (52). Side-by-side comparisons showed both assays to produce equivalent measures of template abundance, and that the absolute

quantification of the mRNA may be unnecessary because constantly transcribed *in vivo* housekeeping genes effectively serve as internal standards for the relative quantification of transcripts of genes of interest (50, 53, 54). My own experience with PCR analysis also supports that conclusion.

Normalization

The normalization of gene expression data is a means to correct sample-to-sample variation in material obtained from different individuals who vary in tissue mass or cell number, RNA integrity, quantity or experimental treatment. Ideally, mRNA levels can be standardized to the cell number but, when employing whole tissue samples, this type of normalization may not be possible (55). Normalization against high quality, accurately measured total RNA (11, 19) was shown to produce biologically relevant quantification results (56). However, this approach is highly dependent on the accurate quantification and quality of the RNA. Nevertheless, normalization against total RNA still does not overcome the problem of various subpopulations. Moreover, total RNA levels may be elevated in highly proliferating cells, which may affect the accuracy of the copy number between normal and tumor cells. Additionally, it is not always possible to quantify total RNA from limited amounts of clinical samples, such as LCM samples (13).

Normalization against 18S RNA has been used (57). Here, too, concerns have been raised regarding rRNA (instead of mRNA) transcription by a different RNA polymerase, potential imbalances in rRNA and mRNA fractions among different samples, and the possible effects of biological factors and drugs on rRNA (58). A great difference in abundance between total RNA and target mRNA could result in different amplification kinetics, which may generate false quantification data. Moreover, rRNA cannot be used for normalization when quantifying targets from polyA-enriched samples (13).

In theory, the use of internal constitutive genes seems the most appropriate normalization standard. A recent publication, which compared 13 endogenous housekeeping genes, identified hypoxanthine ribosyltransferase (*HPRT*) as the single best reference gene to standardize gene expression measurements in tumor tissue (59). However, numerous publications highlight the fact that no single gene is a "gold standard" capable of fulfilling the criteria required of a universal reference gene (*i.e.*, the expression level of the reference gene should remain constant among cells of different tissues and under different experimental conditions) (60).

As an alternative to the use of multiple control genes, different methods for identifying the most suitable combinations of reference genes have been proposed such as ranking reference genes according to the similarity of their expression profiles using a pair-wise comparison, and employing their geometric mean as a normalization factor, based on the assumption that gene pairs showing stable expression patterns relative to each other are appropriate control genes (55). However, this model requires extensive practical validation to identify a combination of reference genes for an individual experiment that will indicate co-expressed genes.

Another model considers not just the overall expression variation, but also systematic variation across sample subgroups (61). Other normalization models exist, but they are neither straightforward nor easy to implement (62, 63). The expression stability of candidate control genes can be determined with either geNorm (55) [http:// medgen.ugent.be/~jvdesomp/genorm] or BestKeeper (39) [http://www.gene-quantification.info]; both formats use Microsoft Excel.

The above discussion emphasizes that normalization remains an unsolved problem, with no one normalization strategy that is applicable to every experimental situation. It is thus up to individual researchers to identify and validate the method most appropriate for their particular experimental conditions (11, 14, 20, 55, 60, 64), to avoid inaccuracies when quantifying gene expression. This situation complicates standardization and its application to the clinical setting.

Additional Confounders and QC Considerations

In addition to variations in PCR reactions, which can affect overall results (*i.e.*, variation in reaction components, thermal cycling conditions, mispairing events at early stages, choice of primers, normalization procedures, assay design, the instrument and reagents used, and human errors), the threshold cycle (C_t or CP) by itself can add to the variation. The C_t is at the heart of any qPCR assay, as it is used to determine the copy number. The C_t is defined as the cycle when the sample fluorescence exceeds a chosen threshold above the calculated background fluorescence. A positive C_t can arise as a result of a true amplification, but some C_t values are not due to true amplification, and some true amplifications do not record a C_t due to the wandering (drifting) baseline caused by an incorrect setting of the background cycle range. In such a situation, an adjustment of the baseline cycle (usually set by the machine algorithm, and not altered in standard runs) to include the lowest point of the amplification plot (adaptive baseline) corrects for this fluorescence drift and allows appropriate recording of the C_t for all samples, including no template control ones (12).

Well-designed primers are essential for optimal results. There are various programs available without charge on the worldwide net for primer design (11, 14): [http://www.broad.mit.edu/genome_software/] allows access to Primer3 primer design software; and a melting temperature (T_m) calculator for optimizing primer design is [http://www.operon.com/oligos/toolkit.php]. Primers must also be validated using the Basic Local Alignment Search Tool

(BLAST) from the National Center for Biotechnology Information [http://www.ncbi.nlm.nih.gov/BLAST/], and is a valuable site for checking the specificity of primer and probe sequences. It is also important to determine whether folding of the mRNA might interfere with primer access during the RT step using the Mfold program [http://www.bioinfo.rpi.edu/applications/mfold/old/rna].

From a quality control perspective, there are generally two sources of sample-to-sample variability in PCR experiments: a) differences caused by variation in the quantity or quality of the samples (*e.g.*, partial degradation or the presence of contaminants), and b) random sample-to-sample variation, which includes user-induced variation. Unfortunately, random variability is a fact in PCR; the best way to minimize it is to run duplicate samples and average the data. Random variability caused by operator error can be minimized by making a cocktail of reagents (*i.e.*, master mix) (2, 8, 23).

Because different people performing the same assay often yield different results, it is paramount that each facility performing real-time qRT-PCR must have available standard operating procedures that are rigorously followed by anyone carrying out this assay.

Recent surveys (14, 65) revealed extensive interlaboratory variations in assay design, validation and analyses that, together with other undesirable practices, are likely to contribute to variable results; all are factors that emphasize the need for standardization of this technology and the adoption of rigorous quality control practices, particularly because of the importance of qRT-PCR as a high throughput diagnostic and prognostic clinical assay.

Application to Colorectal Cancer Diagnosis, Prognosis and Therapy

qRT-PCR has been extensively applied to studying gene expression in carcinogenesis. As exemplified by colorectal cancer (CRC), mRNA expression has been used to develop markers for diagnosis and prognosis in the tissue, stools and blood of patients with adenocarcinoma (23, 66, 67). Additionally, it has been used for the detection of disseminated tumor cells in the peripheral blood of patients with CRC (68), and has been employed in conjunction with LCM to validate CRC microarray expression data (69, 70). Moreover, it has been utilized to study markers that predict response to CRC therapy (71), just to mention a few applications of this technology to a common cancer.

Conclusion

The application of real-time qRT-PCR technology has simplified and enhanced the quantification of DNA and RNA, which has impacted the field of molecular oncology, as large amounts of data can be produced and analyzed in a relatively short period of time. The progressive decrease in the cost of thermal cyclers, as well as the reagents and detection chemistries, promises the increased use of this quantitative technology. Unlike traditional end-point PCR, there are many complexities with real-time PCR that can affect the overall results. However, a well-designed real-time PCR experiment, performed with proper controls, can be one of the most sensitive, effective, fast, accurate, quantitative and reproducible methods of measuring gene expression.

The choice of the particular detection chemistry is dependent upon the characteristics of an individual experiment, although non-specific DNA binding dyes could first be used to optimize the detection conditions, to be followed by sequence-specific, probe-based detection chemistry, to increase the reaction specificity. Quencher technology is now available, which promises to reduce the cost of making specific probes without reduction in detection efficiency.

Although qPCR assays are characterized by high precision and reproducibility, the accuracy of the generated data is largely dependent on other factors such as sample preparation, quality of the standards, choice of appropriate primers and housekeeping gene standards and the normalization procedures. There are computer programs available in the public domain that allow the design optimization of PCR primers. Regarding normalization, the use of multiple housekeeping gene standards promises to increase accuracy. When it is not feasible to employ multiple gene standards, a gene should be chosen which has stable expression and the reference standard chosen should be validated by an algorithm such as geNorm. Combining real-time PCR with powerful techniques such as LCM makes it possible to measure gene expression in specific tumor cells from *in vivo* tissue samples, which allows for meaningful results that will enhance our understanding of the molecular mechanisms of carcinogenesis. Additionally, standardized criteria and international uniformity in the experimental design and data analysis must be established to be able to compare data among different laboratories.

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