



Quality- Controlled Multi-Gene Expression Measurement

An Essential Tool for the Development of Drugs
and Diagnostic Tests

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One of the key expectations of the human genome sequencing project is that multi-gene transcript measurement will accelerate drug development and lead to more accurate diagnostic tests. In keeping with this promise, during the past few years sets of genes have been identified through microarray analysis that are associated with important disease states and/or drug responses (1–6). Identification of these sets of genes has engendered a more acute perception of the need to develop new, sophisticated multi-gene expression measurement methods that are amenable to appropriate quality control. This will be necessary in order to meet FDA guidelines in more advanced stages of drug development, or to develop diagnostic tests that meet Clinical Laboratory Improvement Amendment (CLIA) standards established in conjunction with the Centers for Disease Control (CDC, Atlanta, Georgia, USA). These points were made in recently published FDA guidelines for use of multi-gene transcript measurement methods that optimize the likelihood of new drug application approval by FDA (7). Similarly, CDC is working actively to establish guidelines for using multi-gene transcript measurement methods in diagnostic tests.

In this context, during the past 10 years researchers have developed a multi-gene transcript measurement method, called standardized reverse transcriptase polymerase chain reaction (StaRT-PCR™), in which quality control is an integral part. The key to this method is use of a standardized mixture of internal standards (SMIS™) in each gene expression measurement. Each SMIS contains standards for normalizer genes (including GAPD and β -actin) and 96 target genes. Each transcript is measured relative to its respective internal standard within the SMIS. A sufficient amount of internal standard is prepared for from 100 billion to one trillion assays. Because DNA is stable, this would be a sufficient amount to conduct all gene expression measurements for from 100 to 1000 years at the current rate of gene expression measurement, estimated to be one billion assays a year

The authors describe a multi-gene transcript measurement method that addresses the quality control requirements of FDA and other regulatory agencies. The key to this technique is use of a standardized mixture of internal standards in each gene expression measurement, which allows each transcript to be measured relative to its respective internal standard. A standardized expression database is being developed so that samples can be compared directly, and the presence of an internal standard in every transcript measurement means there are no false negatives and a statistically insignificant number of false positives.

Standardized Expression Measurement

Figure 1. (a) A schematic diagram of the relationship among internal standards within the SMIS and between each internal standard and its respective cDNA from a sample. The internal standard for each normalizer gene and target gene is at a fixed concentration relative to all other internal standards within the SMIS. Within a master mixture, in which a cDNA sample is combined with SMIS, the concentration of each internal standard is fixed relative to the cDNA representing its respective gene. In each sample, the number of cDNA molecules representing a gene is measured relative to its respective internal standard rather than by comparing it to another sample. Because everyone uses the same SMIS, and there is enough to last 100 to 1000 years at the present rate of consumption, all gene expression measurements can be entered into and appropriately compared in the

a. StaRT-PCR			b. Multiplex RT-PCR or microarray	
Sample A	Standardized mixtures of Internal Standards	Sample B _{1-n}	Sample A	Sample B
β -actin	β -actin standard	β -actin	β -actin	β -actin
Gene 1	Gene 1 standard	Gene 1	Gene 2	Gene 2
Gene 2	Gene 2 standard	Gene 2	Gene 3	Gene 3
Gene 3	Gene 3 standard	Gene 3	Gene 4	Gene 4
Gene 4	Gene 4 standard	Gene 4	Gene 5	Gene 5
Gene 5	Gene 5 standard	Gene 5	Gene 6	Gene 6
Gene 6	Gene 6 standard	Gene 6	Gene 7	Gene 7
In each sample, each gene compared to its respective standard within standardized mixture. This enables <ul style="list-style-type: none"> • Inter-sample comparisons • Intra-sample comparisons • Molecules/10^6 ref gene molecules 			Each gene in a sample compared directly to same gene in another sample <ul style="list-style-type: none"> • Inter-sample measurements: Yes • Intra-sample measurements: No • Molecules/10^6 ref gene molecules: No 	

same database. (b) Measurement by multiplex RT-PCR or microarray analysis. Using these methods, each gene scales differently, due to differences in hybridization melting temperatures between cDNA and bound sequence (microarrays) or fluorescent

probes (real-time RT-PCR). Consequently, it is possible to compare relative differences in expression from one sample to another, but not difference in expression among many genes in a sample. Further, it is not possible to develop a reference database, except in relationship

to a non-renewable calibrator sample. Moreover, unless a known quantity of standard template is prepared for each gene, it is not possible to know how many copies of a gene are expressed in the calibrator sample, or the samples that are compared to the calibrator.

(8). Every gene expression measurement made in conjunction with a sample of SMIS can be entered into the rapidly developing Standardized Expression Database™ and then compared directly to other data within the database. This will create a reference database that will be essential for use of gene expression data in clinical diagnostic testing. Due to the presence of an internal standard in every transcript measurement, there are no false negatives and a statistically insignificant number of false positives. Both the reference database and the presence of an internal standard in each measurement simplify quality control required by regulatory agencies.

Thus, in addition to quality control, the best test for a disease state or drug response often comprises the transcript levels of multiple genes, the ideal method should enable multi-gene numerical transcript measurement and mathematical interaction among the multiple values. In addition, because many genes must be measured in a sample, and diagnostic biopsy samples often are very small, the method must function with small amounts of starting cellular material. StaRT-PCR has all of these properties.

How StaRT-PCR Works

StaRT-PCR is a modification of the competitive template (CT) reverse transcriptase method described by Gilliland et al, (9). StaRT-PCR allows rapid, reproducible, standardized, quantitative measurement of data for many genes simultaneously (10–22). An internal standard CT is prepared for each normalizer gene (β -actin and GAPDH) and target gene and normalizer gene (23) (Figure 3), then cloned to generate enough for $>10^9$ assays and carefully quantified. Then, internal standards for the normalizer genes and 96 target genes are mixed together in a SMIS. Each target gene is measured relative to one or more normalizer genes to control for cDNA loaded into the reaction. Also, each target gene and normalizer gene is measured relative to its respective internal standard in the SMIS (Figures 1, 4, 5). Because each target gene and normalizer gene is measured simultaneously relative to a known number of internal standard molecules that have been combined into the SMIS, it is possible to report each gene expression measurement as a numerical value in units of target gene cDNA molecules/ 10^6 normalizer

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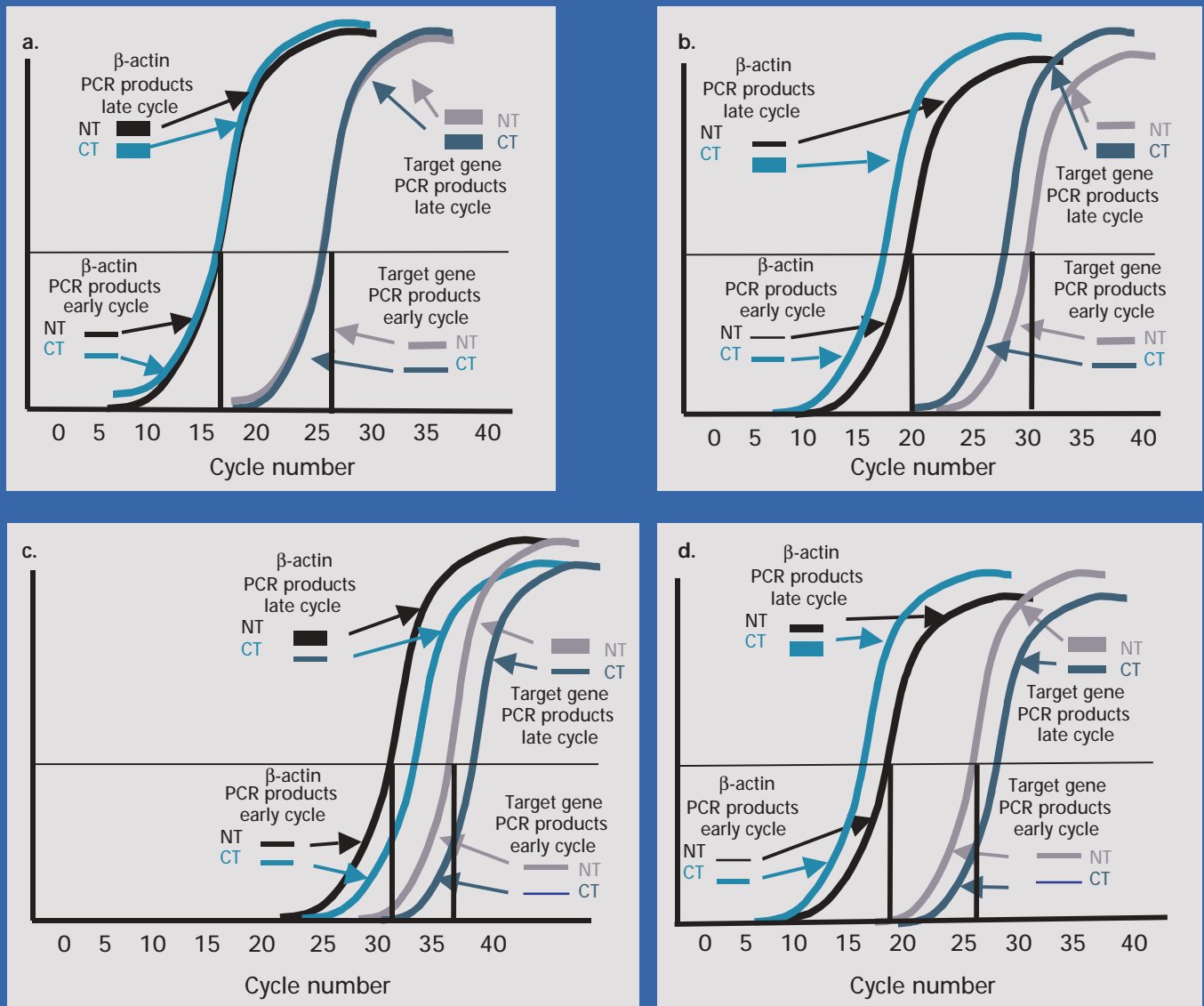


Figure 2. A schematic depiction of simultaneous gene expression measurement by StART-PCR and real-time RT-PCR in two different samples in four different experiments. Shown in each experiment is PCR amplification of a native template (NT) and respective internal standard competitive template (CT) for a target gene and normalizer gene (β -actin).

Although StART-PCR NT and CT products routinely are quantified by densitometry at an endpoint of PCR following electrophoretic separation (as represented by the bands) this schematic demonstrates how the reaction would look if measured at each cycle in real time. For each real-time curve, the CT is represented by a perpendicular black line.

gene cDNA molecules. Calculation of data in this format allows for entry into a common databank (11), direct inter-experimental comparison (10–22), and combination of values into interactive gene expression indices (IGEI) (14, 15, 17).

As shown in the schematic presented in Figure 1a, with StART-PCR expression of each normalizer gene (e.g., β -actin) or target gene (e.g., Gene 1–6) in a sample (e.g., Sample A) is measured relative to its respective internal standard in the SMIS. Because the internal standard for each gene is present at a fixed concentration relative to all other internal standards within an experiment, it is possible to quantify the ex-

pression of each gene relative to all others measured. Further, it is possible to compare data from analysis of Sample A to those from analysis of all other samples represented as B_{1-n} . This results in what could be described as a continuously expanding multiplex experiment (i.e., data from an ever-expanding number of genes and samples can be entered into the same database). Because the number of molecules for each standard is known, it is possible to calculate all data in the form of molecules/normalizer gene molecules.

In contrast, for other multi-gene methods such as multiplex real-time RT-PCR or microarrays (represented in Figure 1b), expression

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Figure 3. Preparation of internal standard competitive templates. (a) Forward (dashed bar) and reverse (solid bar) primers (approximately 20 bp in length) that span a 150–850 bp region are used to amplify the native template (NT) from cDNA. Taq polymerase will synthesize DNA from these primers using the NT (dashed lines). (b) After confirming that native template primers work, a CT primer is designed. This is an approximately 40 bp primer with the sequence for the reverse primer (solid bar) at the 5' end, and a 20 bp sequence homologous to an internal native template sequence (white bar) at the 3' end, collinear with the sequence reverse primer sequence. The 3' end of this 40 bp primer is

designed to be homologous to a region approximately 50–100 bp internal to the reverse primer. The 5' end of this 40 bp primer will hybridize to the region homologous to the reverse primer, while the 3' end will hybridize to the internal sequence. Importantly, Taq polymerase will be able to synthesize DNA using only the primers bound at the 3' end (dashed bar). (c) In the next cycle of PCR, the DNA newly synthesized using the 40 bp primer hybridized to the internal sequence is bound to forward primer (dashed bar), and a homologous strand is synthesized. (d) This generates a double-stranded CT with the reverse primer sequence 100 bp closer to the forward primer than occurs naturally in the NT.

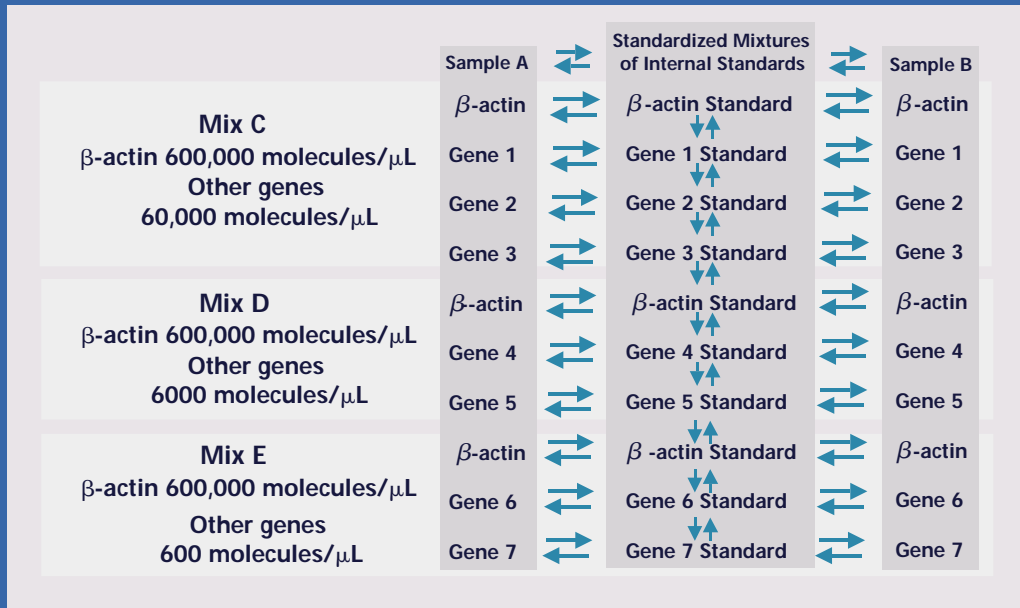
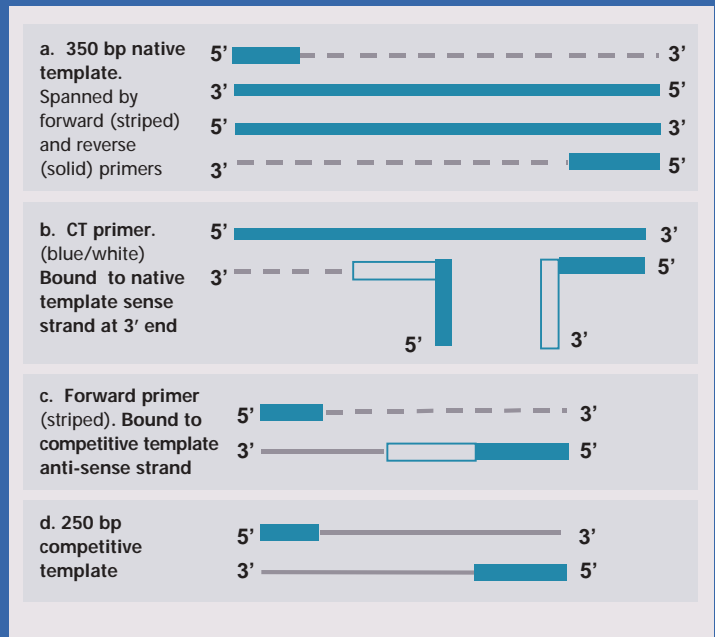


Figure 4. The relationship among mixes serially 10-fold diluted from each 96-gene SMIS. As described in the text, a serial 10-fold dilution of target gene internal standards relative to normalizer gene internal standards (A–F) is prepared for each 96-gene SMIS. This allows StaRT-PCR measurement of each gene even though different genes might be expressed over a range of more than six orders of magnitude.

of each gene is compared directly from one sample to another. Because of inter-gene variation in hybridization efficiency and/or PCR amplification efficiency and the absence of internal standards to control for these sources of variation, it is not possible to directly compare expression of one gene to another in a sample or to obtain values in terms of molecules per molecules of normalizer gene.

Extensively validated in numerous studies, StaRT-PCR can provide both intra- (10–22) and inter-laboratory reproducibility (12) that is suf-

ficient to detect less than two-fold differences in gene expression. StaRT-PCR identified IGELs associated with lung cancer (14–16), pulmonary sarcoidosis (19), cystic fibrosis (20) and chemoresistance in childhood leukemias (17). Furthermore, in a recent multi-institutional study, data generated by StaRT-PCR were sufficiently reproducible to support development of a meaningful gene expression database and, thereby, serve as a common language for gene expression (12).

StaRT-PCR easily is adapted to automated systems and is amenable to the application of

The Standardized Expression Measurement Center

The Standardized Expression Measurement (SEM) Center recently was established at the Medical College of Ohio through a grant from the National Cancer Institute. The SEM Center is in operation and available for use at www.geneexpressinc.com.

As has been described in this article, microarray technology currently is the starting point for most large-scale gene transcript investigations. However, due to limits in lower detection threshold and sensitivity and lack of internal standards, microarray technology is most appropriately applied as a screening tool. For most applications, data obtained through microarray analysis must be validated by a more sensitive and quantitative method. Most investigators use a quantitative RT-PCR method for this purpose.

The purpose of the SEM Center is to provide standardized, reproducible gene expression measurement as a service. The SEM Center achieves these goals by using StaRT-PCR. Further, StaRT-PCR is easily automated and subjected to quality control, which are critical properties for analysis of clinical specimens.

The SEM Center functions similarly to that of a DNA sequencing service. Thus, users send their RNA or cDNA samples to the SEM Center for analysis. Users select a set of genes for measurement and send a requisition listing these selected genes (available at the SEM Center website) along with the samples.

Technology Incorporated by the SEM Center

Automated preparation of StaRT-PCR reactions. A PerkinElmer (Boston, Massachusetts, USA) robotic liquid handler is used to prepare 10 μ L PCR reactions in 96- or 384-well microplates. First, the liquid handler is programmed to distribute 1 μ L of primers for the requested genes into wells of the microplates. Second, for each cDNA, a sufficient volume of PCR mixture for the anticipated number of gene expression measurements is prepared, containing buffer, Taq polymerase, dNTPs, cDNA and internal standards. The robot then distributes 9 μ L of this PCR

reaction mixture into each well. Thus, in each well, the internal standard CTs for each gene and cDNA are present in the same ratio. However, because only one pair of primers is present in each well, only one gene and its respective internal standard CT are amplified in each well. Following 35 cycles of PCR, each microplate is transferred to a AMS 90 SE30 high-throughput microfluidic device (Caliper/Zymark, Hopkinton, Massachusetts, USA) for analysis.

Isolation and quantification of StaRT-PCR products. Theoretically, the SMIS prepared for StaRT-PCR can be used to measure gene expression using any method capable of quantifying strands of DNA with different sizes, or even single base-pair differences. Below are some of the known methods that can be used. It is possible, even likely, that additional high-throughput methods will be established in the future.

Electrophoretic separation. When StaRT-PCR first was developed, products were separated on agarose gels (10, 11). This method is reliable but relatively costly, time consuming and labor intensive. Through advances in capillary electrophoresis (CE), much faster and less expensive methods for separation of StaRT-PCR products have become available. We compared separation of StaRT-PCR products on agarose gel, the PerkinElmer 310 CE and the 2100 Bioanalyzer microfluidic CE (Agilent, Santa Clara, California, USA) (25). Each of these methods provided results that were statistically the same. Presently, the most robust way to quantify StaRT-PCR products is to electrophoretically separate and quantify endpoint PCR products using the AMS90 SE30. Using a single AMS90 SE30, it is possible to conduct more than 1000 StaRT-PCR assays in eight hours.

The SEM Center employs a microfluidic chip with a sipper that moves from well to well of a microplate, aspirating and then electrophoretically separating StaRT-PCR products every 30 seconds. This allows analysis of a 384-well plate in approximately three hours, which is comparable to the throughput of real-time

quality control procedures. Recently, we established the National Cancer Institute (Bethesda, Maryland, USA)-funded Standardized Expression Measurement (SEM) Center at the Medical College of Ohio (Toledo, Ohio, USA), which uses robotic systems to conduct high-throughput StaRT-PCR gene expression measurement (see sidebar). In the SEM Center, the coefficient of variation for StaRT-PCR is less than 15% for all genes and less than 10% for most genes.

In the following section, we describe in detail the StaRT-PCR method and compare and contrast StaRT-PCR to real-time RT-PCR, which is a well-established quantitative RT-PCR method. In our sidebar, we describe the SEM Center, in-

cluding the equipment and methods used, how to access it and the type of data produced.

StaRT-PCR Versus Real-Time RT-PCR

Several potential sources of variation exist in quantitative RT-PCR gene expression measurement, as outlined in Table I. StaRT-PCR, by including internal standards in the form of a SMIS in each gene expression measurement, controls for each of these sources of variation. The advantages of this approach have been cited independently (24). In contrast, using real-time RT-PCR without internal standards, it is possible to control for some, but not all, of these sources of variation. Furthermore, with real-time RT-PCR, control often requires ex-

devices. A typical electropherogram and quantification calculation is presented in Figure 5.

Other methods. Size separation and quantification. Other methods that may be used to quantify PCR products based on size separation include high-performance liquid chromatography (HPLC) and gas chromatography.

MALDI-TOF. Quantification of gene expression through analysis of RT-PCR products by matrix-assisted laser desorption ionization-time-of-flight mass spectrometry (MALDI-TOF MS) recently was described (8). MALDI-TOF analysis of StaRT-PCR products will have the previously described advantages over analysis of conventional RT-PCR products, due to the use of SMIS. The SMIS already was prepared by Gene Express.

Solid-phase hybridization: microarrays and microbeads. We recently developed and submitted for patent approval a method for analysis of Sta-RT-PCR products on microarrays or microbeads.

Real-time RT-PCR. Based on success with analysis of StaRT-PCR products with solid-phase hybridization to sequences specific for NT or CT products, it will be straightforward to develop fluorescent probes for use of SMIS in real-time RT-PCR.

Summary of methods for analysis of StaRT-PCR products. At the present time, analysis of StaRT-PCR products on the AMS90 SE30 offers the most robust, high-throughput option for use in a service center. However, analysis by microarrays, microbeads, MALDI-TOF MS and/or real-time RT-PCR are likely to have important applications for particular purposes. When StaRT-PCR products, obtained in the presence of SMIS, are analyzed by those methods, they will be directly comparable to results obtained following analysis on the AMS90 SE30. This is supported by preliminary data confirming that we obtain the same result when we quantify StaRT-PCR products by Caliper AMS90 SE30

electrophoretic separation or by hybridizing them to specially designed standardized microarrays.

Design of high-throughput StaRT-PCR experiments. All of the genes that are to be measured in a given sample will be measured simultaneously. Due to the presence of the SMIS in every PCR reaction, gene expression values for one sample can be compared to gene expression values from another sample evaluated in a second experiment later the same day or another day (Figure 1a).

PCR products (NT and CT) for as many as four genes can be electrophoresed (separated and quantified) in the same microfluidic channel of the AMS 90 SE30. Accomplishing this in the high-throughput SEM Center requires software that identifies which genes can be electrophoretically separated simultaneously; based on the length in base-pairs of the NT and CT PCR for each gene, the primers and CTs are designed to amplify PCR products that range from 150 to 850 bp. Thus, for every set of genes to be analyzed, the software must identify which genes may be electrophoresed together.

Standardized Gene Expression Database. Users send samples to the SEM Center without any annotating information and with a requisition that includes an attestation that any primary human samples were obtained under approved and active investigative review board (IRB) protocol. Because no potentially identifying information is provided, the SEM Center is exempted from the need to obtain an IRB protocol for each set of samples submitted. As soon as an order is completed, the data are sent by email and hard copy to the user. Each user is encouraged to send the annotating information as soon as they are comfortable doing so. It is hoped that users will send the annotating information as soon as a manuscript containing the data is accepted for publication, or sooner. An annotated standardized gene expression database will be key both for advances in research as well as for developing clinical tests.

ternal standard curves, and these add times and are themselves a potential source of error.

Figure 2 is a schematic presentation of the way quantitative measurements are made in the two forms of quantitative RT-PCR discussed here: real-time RT-PCR and StaRT-PCR. In real-time, the fluorescent PCR product is measured at each of the 35 to 40 cycles. If four different fluorophores are used, as many as four PCR products can be monitored simultaneously in real-time. In Figure 2a, the native template (NT) and CT for β -actin and the NT and CT for the target gene are PCR-amplified simultaneously. In StaRT-PCR, the products of endpoint PCR are electrophoretically separated, and for each gene

the shorter CT PCR product migrates faster than the NT PCR product. The PCR products are electrophoresed in the presence of fluorescent intercalating dye and densitometrically quantified. If there is more NT product than CT product, the NT band will emit more fluorescent light; if there is more CT product than NT product, the CT band will emit more fluorescent light. Importantly, the ratio of NT/CT that is present at the beginning of PCR will remain constant throughout PCR to endpoint. For this reason, with StaRT-PCR it is not necessary to monitor the PCR reaction in real-time to ensure that the reaction is in log-linear phase (Figure 2a). In addition, measurement of both a normalizer and

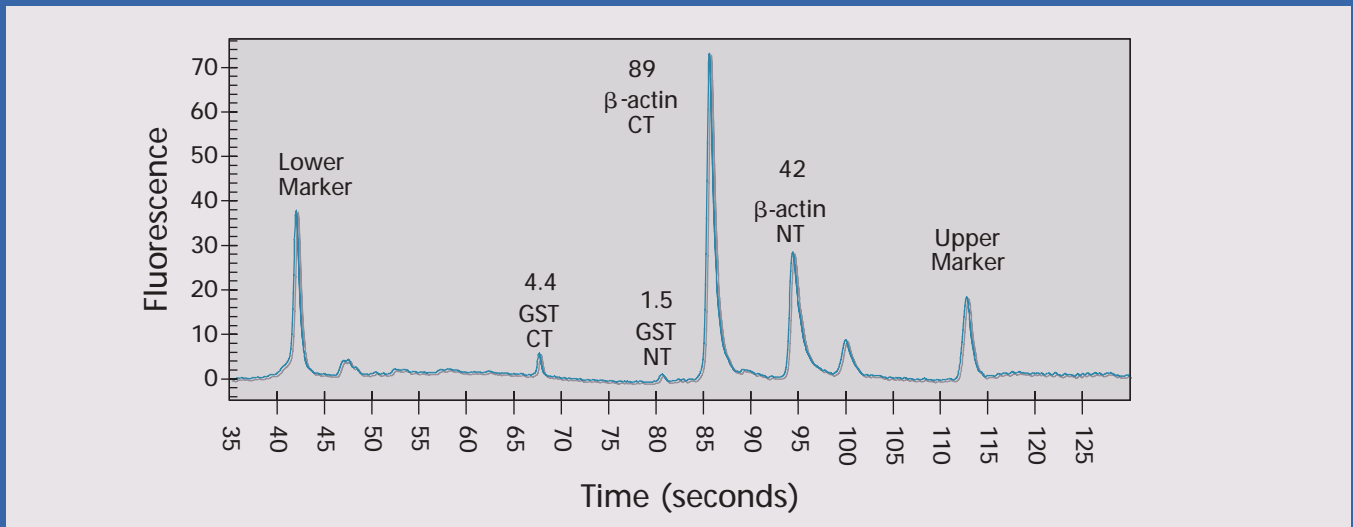


Figure 5. Calculations involved in StaRT-PCR measurement of GSTT gene expression relative to β -actin in an actual bronchial epithelial cell (BEC) sample. The native template (NT) PCR product was amplified from cDNA specific

for the gene being measured, and the competitive template (CT) PCR product was amplified from the internal standard for each respective gene. A volume of SMIS containing a known number of internal standard CT

molecules for β -actin (600,000) and GSTT (6000) was included at the beginning of the PCR reaction. For each gene, the NT and CT amplify with the same efficiency, the β -actin gene NT/CT PCR product ratio allows

determination of the number of β -actin NT copies at the beginning of PCR and the target gene NT/CT ratio allows determination of the number of target gene NT copies at the beginning of PCR.

a target gene in every PCR reaction controls for loading from one sample to another (Figure 2b) or among replicate measurements of the same sample (Figure 2d). Further, the use of a SMIS ensures that the concentration of internal standard for each gene relative to all others remains fixed in each experiment. With StaRT-PCR, variation in PCR amplification efficiency caused by the presence of an inhibitor in the sample, an inhibitor in the PCR reaction vessel, defective PCR reagent or wrong concentration of a PCR reagent is controlled for by the presence of internal standards in every PCR reaction.

For the experiment demonstrated in Figure 2a, sample 1 was evaluated and there were about equivalent copies of β -actin NT and CT present at the beginning of the PCR reaction. Thus, following electrophoresis of the β -actin PCR products, the NT and CT bands are about equivalent, and during real-time measurement, the fluorescent intensity for the NT will be about the same as for the CT. The NT/CT ratio is the same at an early cycle as it is at a late cycle (endpoint). Similarly, the target gene NT band and CT band are about equivalent, and the real-time value for the NT is about the same as for the CT. The ΔC_T between β -actin and the target gene is about 10. Methods for calculating numeric value for target gene expression using StaRT-PCR are presented in Figure 5. In Figure 2b, we see repeat analysis of sample 1 but with a lower amount of cDNA loaded, due to a variation in pipetting.

The NT/CT ratio for β -actin is lower, but because the relative concentration of each CT is fixed in the SMIS and the relative representation of each gene in the cDNA is fixed, the NT-CT ratio for the target gene goes down commensurately, so that the odds ratio of target gene NT-CT divided by normalizer gene NT-CT and gene expression value, in terms of molecules/ 10^6 β -actin, remains the same as in Figure 2a. Correspondingly, the ΔC_T in real-time analysis is unchanged. In Figure 2c, we see repeat analysis of sample 1, but with both a larger amount of cDNA loaded due to variation in pipetting and gene-selective low efficiency PCR, as might be caused by inhibitor in the sample, inhibitor in the well or inappropriate concentrations of normalizer gene primers. As a consequence of the gene-selective low efficiency, ΔC_T is reduced from 10 to 6. Thus, in real-time measurement, the gene selective inhibition would be associated with a decreased ΔC_T and erroneous measurement. However, in StaRT-PCR, both the variation in loading and variation in efficiency are controlled for by internal standards for normalizer gene and target gene in a SMIS. With the larger amount of cDNA loaded, the β -actin NT/CT ratio and the target gene NT/CT ratio increase commensurately. Because there is no change in NT/CT ratio for either normalizer or target gene, the odds ratio of target gene NT/CT divided by the normalizer gene NT/CT stays the same as in Figures 2a and 2c. Looking at Figure 2d, for sample 2,

Standardized Expression Measurement

Table I. Sources of Variation in Quantitative RT-PCR Gene Expression Measurement and Control Methods

Source of Variation	Control Methods	
	StaRT-PCR ¹	Real-time
cDNA loading: Due to variation in pipetting, quantification, reverse transcription. Consequence: unreliable comparison of expression for same gene in two different samples	Multiplex Amplify with Reference Gene (e.g. β -actin)	Multiplex Amplify with Reference Gene (e.g. β -actin)
Amplification Efficiency Cycle-to-Cycle Variation: early slow, log-linear, and late slow plateau phases Consequence: unreliable comparison of expression for same gene indifferent samples	Internal standard CT for each gene in a Standardized Mixture of Internal Standards (SMIS)	Real-time measurement
Gene-to-Gene Variation: in efficiency of primers Consequence: unreliable comparison of expression for different genes in the same or different samples	Internal standard CT for each gene in a SMIS	External standard curve for each gene measured
Sample-to-Sample Variation: variable presence of an inhibitor of PCR Consequence: unreliable comparison of expression for same or different gene in same or different samples	Internal standard CT for each gene in a SMIS	Standard curve of reference sample compared to test sample ²
Reaction-to-Reaction Variation: in quality and /or concentration of PCR reagents (e.g. primers) Consequence: unreliable comparison of expression for same or different genes in same or different samples	Internal standard CT for each gene in a SMIS	None ²
Reaction-to-Reaction Variation: in presence of an inhibitor of PCR Consequence: unreliable comparison of expression for same or different gene in same or different samples	Internal standard CT for each gene	None ²
Position-to-Position Variation: in thermocycler efficiency Consequence: e.g., unreliable comparison of expression for same or different gene in same or different samples	Internal standard CT for each gene	None ²

¹StaRT-PCR involves a) the measurement at end-point of each gene relative to its corresponding internal standard competitive template to obtain a numerical value and b) comparison of expression of each target gene relative to the β -actin normalizer gene, to obtain a numerical value in units of molecules/ 10^6 β -actin molecules. Use of normalizer genes other than β -actin is discussed in text.

²Variation in the presence of an inhibitor in a sample can be controlled through use of standard curves for each gene in each sample measured and comparing these data to data obtained for each gene in a "calibrator" sample. However, variation in PCR reaction efficiency due to inhibitors in samples, variation in PCR reagents or variation in position within the thermocycler can be compensated for only through use of an internal standard for each gene measured in the form of an SMIS. If an internal standard is included in a PCR reaction, quantification can be made at end-point, and there is no need for kinetic (or real-time) analysis. If internal standards for multiple genes are mixed together in an SMIS and then used to measure expression for both the target genes and normalizer gene, this is the patented StaRT-PCR technology, whether it is done by kinetic (real-time) analysis or at endpoint. An SMIS fixes the relative concentration of each internal standard so that it cannot vary from one PCR reaction to another, whether in the same experiment or in another experiment, on another day, in another laboratory.

the target gene is expressed at a higher level than in sample 1 and ΔC_T is about 7. There were fewer copies of β -actin NT than CT present at the beginning of the PCR reaction. Thus at the end of PCR, the electrophoretically separated β -actin NT band is less dense than the CT band, and throughout real-time measurement, the fluo-

rescence value of the NT is less than that of the CT. However, even though less sample 2 cDNA was loaded into the PCR reaction compared to sample 1, the target gene NT band is more dense than the target gene CT band, and the target gene NT fluorescence value during real-time measurement is higher throughout PCR.

Thus, with real-time RT-PCR, it is possible to control loading by measuring the target gene and normalizer gene in the same PCR reaction (Figures 2a, b, d). The C_T (for each gene represented by a black line intersecting with the X axis) for the normalizer gene and the target gene both could vary from one experiment to another, but the ΔC_T will not vary. However, real-time might not control for well-to-well variation in the presence of inhibitors, or the quality of PCR reagents or sample-to-sample variation in PCR efficiency due to inhibitors (e.g., heme) (Figure 2c). Presence of an inhibitor could alter PCR amplification efficiency of one gene compared to another (25). A bad lot or inappropriate concentration of primers for the normalizer gene or the target gene would cause variation in PCR amplification of one gene relative to another. As depicted in Figure 2c, amplification efficiency of the normalizer gene in sample 1 is affected by low concentration of primer, but amplification efficiency of the target gene is normal. The result is that the DCT is reduced from 10 in Figure 2a to 6 in Figure 2c, and the value for expression of the target gene is inappropriately high. In contrast, with StaRT-PCR, because for each gene the amplification efficiency of the internal standard CT is affected the same way as the NT, the ratio is unchanged in Figures 2a and 2c for either normalizer gene or target gene, and using the ratio of NT/CT for target gene relative to NT/CT for normalizer gene controls for variation in amplification efficiency.

Summary

This article has described how StaRT-PCR can be used to conduct multi-gene transcript measurements that address the quality control requirements of FDA and other regulatory bodies. The key to technique is the use of a standardized internal standards in each gene expression measurement, which means that each transcript is measured relative to its respective internal standard. Furthermore, every gene expression measurement made using these standards can be entered into the rapidly developing database for direct comparison with other data within the database. This will create a reference database that will be essential for use of gene expression data in clinical diagnostic testing. Both the reference database and the presence of an internal standard in each measurement simplify quality control required by regulatory agencies.

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