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Sensitivity and reproducibility of standardized-competitive RT-PCR for transcript quantification and its comparison with real time RT-PCR

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Abstract

Background: Probe based detection assays form the mainstay of transcript quantification. Problems with these assays include varying hybridization efficiencies of the probes used for transcript quantification and the expense involved. We examined the ability of a standardized competitive RT-PCR (StaRT PCR) assay to quantify transcripts of 4 cell cycle associated genes (RB, E2F1, CDKN2A and PCNA) in two cell lines (T24 & LD419) and compared its efficacy with the established Taqman real time quantitative RT-PCR assay. We also assessed the sensitivity, reproducibility and consistency of StaRT PCR. StaRT PCR assay is based on the incorporation of competitive templates (CT) in precisely standardized quantities along with the native template (NT) in a PCR reaction. This enables transcript quantification by comparing the NT and CT band intensities at the end of the PCR amplification. The CT serves as an ideal internal control. The transcript numbers are expressed as copies per million transcripts of a control gene such as β-actin (ACTB).

Results: The NT and CT were amplified at remarkably similar rates throughout the StaRT PCR amplification cycles, and the coefficient of variation was least (<3.8%) when the NT/CT ratio was kept as close to 1:1 as possible. The variability between the rates of amplification in different tubes subjected to the same StaRT PCR reaction was very low and within the range of experimental noise. Further, StaRT PCR was sensitive enough to detect variations as low as 10% in endogenous actin transcript quantity ($p < 0.01$ by the paired student's *t*-test). StaRT PCR correlated well with Taqman real time RT-PCR assay in terms of transcript quantification efficacy ($p < 0.01$ for all 4 genes by the Spearman Rank correlation method) and the ability to discriminate between cell types and confluence patterns.

Conclusion: StaRT PCR is thus a reliable and sensitive technique that can be applied to medium-high throughput quantitative transcript measurement. Further, it correlates well with Taqman real time PCR in terms of quantitative and discriminatory ability. This label-free, inexpensive technique may provide the ability to generate prognostically important molecular signatures unique to individual tumors and may enable identification of novel therapeutic targets.

Background

Molecular definition of tumors can be achieved at the genomic, transcriptomic or proteomic level. Morphologically similar tumors are often prognostically distinct and the different clinical outcomes are due to differences in biology. Molecular pathology is therefore rapidly evolving to include characterization of unique molecular patterns in tumors. While genomic changes are crucial in the initial stages of a disease, dynamic alterations in the expression of these genes at mRNA and protein levels determine the disease progression. As the evidence for involvement of intricate molecular pathways in disease progression continues to rapidly accumulate, relational transcript quantification for coordinately linked genes will provide a novel tool for tumor characterization. These transcript variations can be characterized as fluctuations in expression of a given gene across many tumors (*expression profile*) or changes in expression of many genes in the same tumor (*expression signature*) [1]. A number of methods are available for quantitative transcript measurement, including cDNA microarrays and real time RT-PCR. Until recently, however, there was no method available that could easily be subjected to quality control, which is critical for clinical diagnostic and pharmaceutical industry applications. Further, all previously available methods were limited to generating a 'profile', and not fine-tuned for assigning a molecular 'signature', because they did not control for differences in efficiency of hybridization for different gene probes with their corresponding cDNA. In contrast, competitive RT-PCR has the capability to achieve hybridization-independent transcript quantification through the incorporation of internal competitors nearly identical to the native cDNA templates in the reaction mixture. This technique has received only limited attention in the past few years because of the lack of standardization. Recently, however, this technique has been standardized by designing and incorporating competitors at precise copy numbers for all individual genes to be quantified [2]. The competitive templates thus provide the internal standards and allow end point PCR quantification. The Standardized RT-PCR (StaRT PCR) technique quantifies transcripts by comparing the NT and CT band intensities at the end of the PCR amplification and the transcript numbers are expressed as copy numbers per million molecules of a housekeeping gene such as β-actin. Further, StaRT PCR has the ability to reliably quantify variations in expression of different genes, as long as internal standards and endogenous targets amplify with identical efficiency. StaRT PCR is thus capable of generating both molecular profiles and signatures, is relatively inexpensive, and has the potential to develop into a high-throughput assay system for transcript quantification [2].

In this paper, we have examined the ability of StaRT PCR to quantify transcripts of 4 cell cycle-associated genes (RB, E2F1, CDKN2A and PCNA) in two cell lines and compared it with the established real time RT-PCR assay. Two different bladder tissue-derived cell lines were used – T24 (an established commercially available urothelial carcinoma cell line) and LD419 (a primary culture fibroblastic cell line). Further, we demonstrate the sensitivity, reproducibility and consistency of this technology, testing for variables such as cell type, culture confluence and starting total RNA amount.

Results

Linearity of Amplification

When the observed NT/CT ratio for each assigned NT/CT ratio was plotted against the number of cycles when amplification was terminated, the NT/CT ratio was found to remain relatively constant regardless of the amplification end point (figure 1), indicating that the NT & CT were amplified at a comparable rate throughout the exponential phase of Start PCR amplification process.

For each designed NT/CT ratio, coefficients of variation (CV) between the initial ACTB transcript numbers in the NT were calculated from the observed NT/CT ratios at the end of StaRT PCR amplification. CV between the estimated ACTB transcript numbers at the different NT/CT ratios was found to be the least (3.8%) when the ratio was kept close to 1. (Table 1) The CV between the estimated ACTB transcript numbers was fairly low (<16%) over a wide range of NT/CT ratios (0.1 to 10.0). The results were comparable for similar experiments performed for the 3 other genes analyzed in this study (RB1, E2F1, and PCNA – data not shown), except for CDKN2A, where it was not possible to calculate the CV since the expression levels were too low to be detected by gel electrophoresis until cycle number 32.

Inter-sample variability

ACTB transcripts (from LD 419 cDNA), amplified in multiple tubes simultaneously, were quantified separately in the PCR reactions terminated at cycles 20, 23, 26, 29, 32 & 35, and the variability between the values obtained from the multiple tubes were compared. The variability (coefficients of variation) between the transcript numbers from the multiple tubes ranged from 0.70% to 5.28%, and was within the range of inter-assay variability observed by real time RT-PCR method [4]. (Table 2)

Ability of StaRT PCR to quantify small changes in gene expression

NT/CT ratios were designed to test the ability of StaRT PCR to quantify minute changes in gene expression. The ability of the assay system to detect changes in the amount of the native template between consecutive NT/CT ratios was assessed using the paired student's *t*-test. StaRT PCR

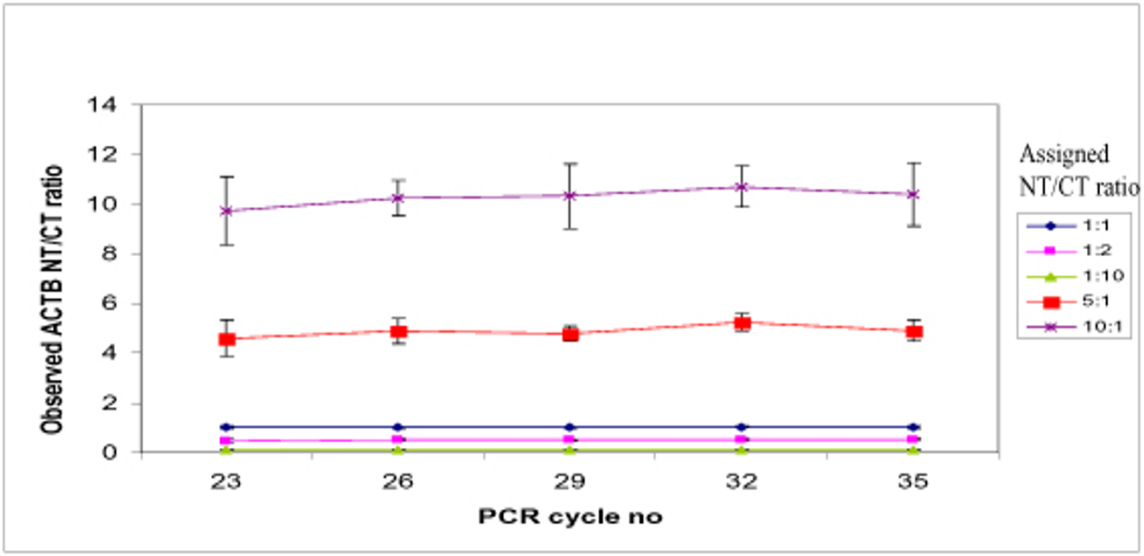

Figure 1

Determination of linearity of NT/CT ratios over PCR cycles from 23–35: LD419 cDNA was mixed with the CT to arrive at NT/CT ratios: 0.1, 0.5, 1, 5, and 10. Independently, each of these 5 initial NT/CT ratios were used to set up separate reactions, which were then subjected to PCR amplification with ACTB primers and terminated at cycles 23, 26, 29, 32, 35. All experiments were performed in triplicate. The mean observed NT/CT ratios (following quantification using Bio-Rad Quantity One software) at each end point were plotted against the cycle numbers at which the reactions were terminated. The observed NT/CT ratios remain significantly constant during the StaRT PCR amplification cycle when the assigned NT/CT ratio is close to 1.

Table 1: Determination of Coefficients of Variation (CV) to examine variability in transcript numbers for different NT/CT ratios: The CV between the initial ACTB transcript number in the NT was calculated based on the observed NT/CT ratios at the end of StaRT PCR amplification. LD419 cDNA was mixed with CT to obtain assigned NT/CT ratios: 0.1, 0.5, 1, 5, and 10. Independently, each of these 5 assigned NT/CT ratios was used to set up separate reactions, which were subjected to PCR amplification. All experiments were performed in triplicate. The initial number of ACTB transcripts at each assigned NT/CT ratio was calculated at the end of the StaRT PCR amplification. CV between the calculated ACTB transcript numbers at the different NT/CT ratios was found to be the least when the ratio was kept close to 1. Further, the CV between the calculated ACTB transcript numbers was fairly low over a wide range of NT/CT ratios.

Assigned Actin NT/CT Ratio	Mean Observed Actin NT/CT Ratio	Coefficient of Variation (= 100% × s.d./mean)
0.1	0.101	15.8%
0.5	0.614	9.3%
1.0	1.01	3.8%
5.0	4.68	13.4%
10.0	9.23	8.7%

Table 2: Comparison of inter-tube variability in transcript quantification: ACTB transcripts were amplified in multiple tubes simultaneously and the PCR reactions terminated at cycles 20, 23, 26, 29, 32 & 35. Experiments were performed in triplicate. The ACTB transcript number in all tubes was quantified separately at the designated end points and the variability between the values obtained from the multiple tubes compared. There is very little variation between the transcript numbers obtained from the multiple tubes at each end point.

Number of cycles of amplification	Mean transcript number from 3 tubes	Standard deviation	Coefficient of variation
20	150369	3012.87	2%
23	144224	3047.39	2.11%
26	139305	7354.84	5.28%
29	135205	996.66	0.70%
32	135138	2175.03	1.61%
35	135413	2797.04	2%

was sensitive enough to detect even the least tested change (7%) in the transcript amount (figure 2, $p < 0.01$).

Furthermore, although they represent different ranges of ACTB transcript estimates, both LD419 and T24 titration curves have identical slopes, indicating the consistency of the technique to quantify transcripts, regardless of different starting transcript numbers. A linear regression analysis was used to summarize and evaluate the relationship between the serial NT/CT ratios and the number of transcripts estimated. In an analysis of variance, lack of linearity was tested using the lack-of-fit sums of squares. The error sum of squares with the dilutions (11 levels) was subtracted from the error sum of squares for the simple linear regression model and this was compared to the error sum of squares in the analysis of variance. Upon analyzing for the T24 and LD419 cell lines separately, the slope of the fitted line was used to estimate the average increase in number of transcripts per unit change in the NT/CT ratio (figure 2). Using the simple linear regression, we estimated that for every 0.1 unit of increase in the "NT/CT ratio (e.g., from 0.8 to 0.9 or from 1.1 to 1.2), the number of β-actin transcripts in the T24 cell line increased on average by 39,062 +/- 1,412, and for the LD419 cell line, on average by 55,656 +/- 1,491. While the examination of the plots, suggested that the plot for LD419 was not truly linear towards the ends (figure 2, $p < 0.001$ for LD419, as compared to $p = 0.14$ for T24), the patterns were consistently linear for the central part (for NT/CT ratios from 0.7 to 1.3), ($p = 0.41$ for LD419 and $p = 0.64$ for T24) and almost uniform increases in the number of transcripts were estimated. Thus, for every 0.1 increase in the NT/CT ratio, the average number of β-actin transcripts increased by 34,076 +/- 2,095 for the T24 cell line and by 48,871 +/- 2,466 for the LD419 cell line. This fairly uniform increment in the transcript number estimation between the consecutive NT/CT ratios indicates that the quantification faithfully followed the serial NT/CT titrations. This also demonstrates the ability of StaRT PCR to accurately and reliably detect changes in transcript quantity as low as 7%.

Correlation between Start PCR and real time PCR

While StaRT PCR quantifies gene expression as the absolute number of target transcripts per million ACTB transcripts using end point analysis, real time PCR uses an exponential phase measurement to quantify target gene expression using the normalized reporter fluorescence intensity of the target transcript at a threshold cycle number (Ct). The values for the numbers of transcripts were log transformed prior to the analysis to correct for heteroscedasticity. Due to the intrinsic differences in the assay systems, scatter plots of the ranked values and the Spearman rank correlation coefficient were used to compare the two methods. The transcript numbers for each gene quantified by both methods at the chosen experimental conditions (2 cell lines, 2 confluence levels, 3 initial RNA amounts-all in triplicate, a total of 36 values) were ranked by relative transcript abundance and compared. Fisher's z-transformation of the correlation coefficient was used to construct 95% confidence intervals for the correlation coefficient. The Spearman rank correlation data (Table 3) demonstrates that the two methods resulted in comparable rankings for expression for each of the four genes.

Discriminatory analysis showed that both assay systems were equally capable of discriminating between the two cell lines based on the expression level of the four chosen genes (Table 4). Further, confluence patterns contributed to a definite stratification of expression values of some of the chosen genes by either method.

Results of the F-test showed that, confluence contributed to stratifying the Rb gene expression levels in both cell lines as detected by StaRT PCR, while real time PCR could not detect this effect. Real time PCR data, however, indicated confluence to be a factor stratifying E2F1 gene

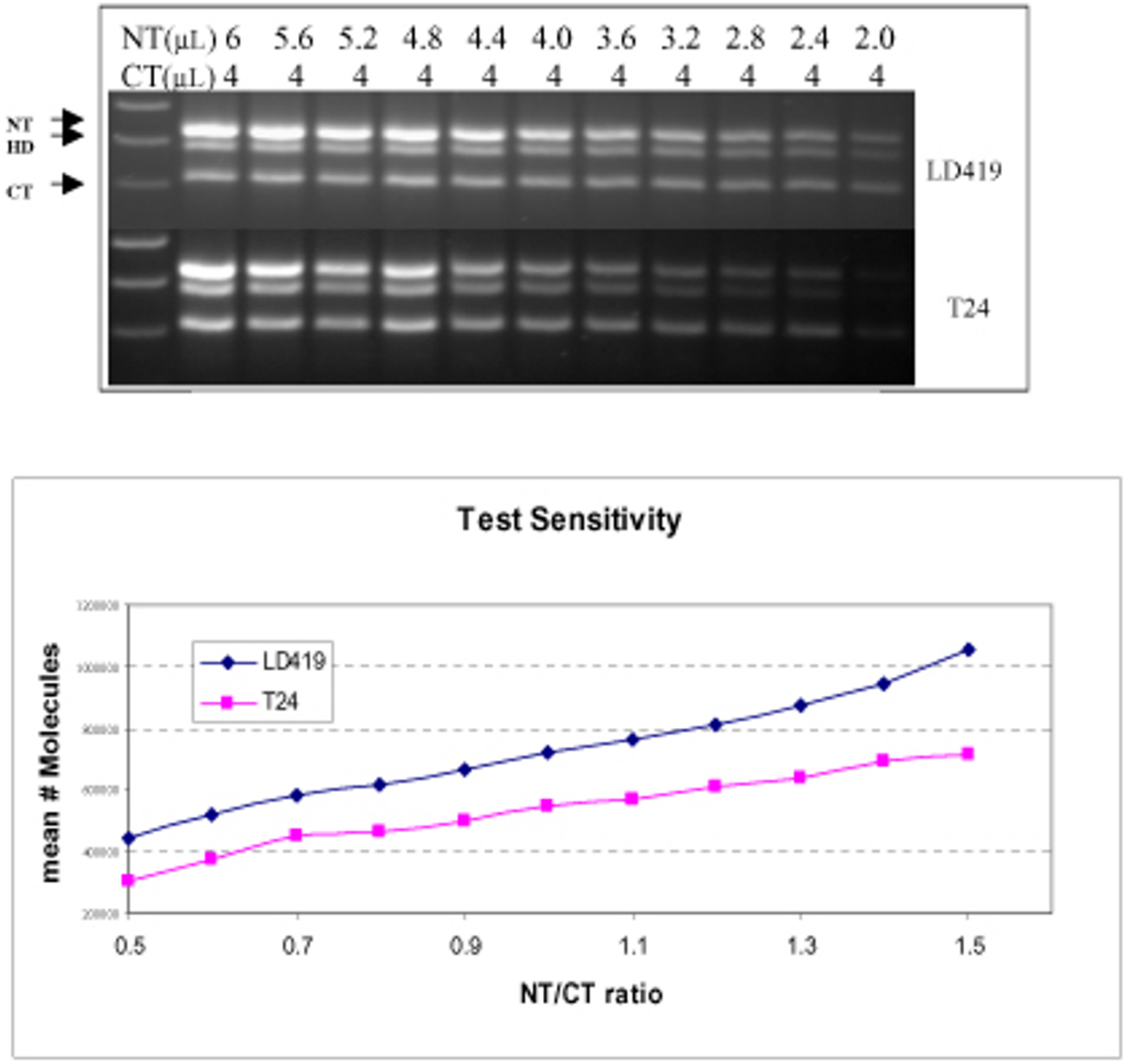

Figure 2

Ability of StaRT PCR to detect minute variations in transcript amounts. NT/CT ratios were designed to test the ability of StaRT PCR to detect minute changes in transcript quantity. cDNAs from LD419 and T24 cells were normalized using ACTB primers. The starting cDNA concentrations used for this experiment were different for T24 (equivalence point) and LD419 (approximately 1.5 × equivalence point). NT volumes decreasing successively by 0.4 µL were mixed with a constant volume of CT (4 µL of 10–12 M ACTB) to obtain NT/CT ratios over a range of 1.5:1 to 0.5:1. This allowed us to test the sensitivity of StaRT PCR over a range of 7%–15%. These reaction mixes were then subjected to StaRT PCR amplification using ACTB primers and the corresponding number of ACTB transcripts in the NT was calculated. The absolute number of ACTB transcripts quantified at each NT/CT ratio was plotted against the NT/CT ratio. StaRT PCR was sensitive enough to detect even the least tested change (7%) in the transcript amount ($p < 0.01$ using the paired Student's *t*-test).

Table 3: Comparison between the transcript quantification efficiencies of StaRT-PCR and Real Time PCR: Due to the intrinsic differences between the two techniques of transcript quantification, the Spearman rank correlation method was used to compare the transcript quantification efficiencies for the two methods. The transcript numbers for each gene quantified by both methods at the chosen experimental conditions (2 cell lines, 2 confluence levels, 3 initial RNA amounts-all in triplicate, a total of 36 values) were ranked by relative transcript abundance and compared. p values <0.05 are considered significant. High correlation between both methods was observed.

Spearman correlation between StaRT-PCR and Real Time PCR methods with 95% CI			
Markers	**Correlation**	**95% CI***	*p* value
Rb	0.679	(0.451–0.824)	<0.01
E2F1	0.707	(0.493–0.840)	<0.01
p16	0.781	(0.609–0.883)	<0.01
PCNA	0.829	(0.688–0.910)	<0.01

*Based on Fisher's transformation of the correlation coefficient

Table 4: Comparison between StaRT PCR and Real Time PCR techniques for their discriminatory power: The ability of the 2 techniques to distinguish between different confluence patterns and starting total RNA amounts based on the expression of individual genes was examined. All experiments were performed in triplicate. The numbers represent p values & values <0.05 (values in bold) are considered significant. Both StaRT PCR and Real Time PCR techniques have comparable power to discriminate between transcriptional status of cultures cells based on transcript quantification. As hypothesized, the differences in the starting RNA amount did not influence the transcript quantification.

	Rb		E2F		p16		PCNA	
	StaRT PCR	Real Time PCR	StaRT PCR	Real Time PCR	StaRT PCR	Real Time PCR	StaRT PCR	Real Time PCR
LD 419 Confluence 60% vs. 100%	**0.019**	0.33	0.36	0.31	0.62	0.21	**<0.001**	**0.023**
LD 419 Starting RNA amount	0.31	0.83	0.3	0.58	0.48	0.95	0.8	0.94
T24 Confluence 60% vs. 100%	**<0.001**	0.4	0.18	**0.024**	0.061	0.99	0.73	0.86
T24 Starting RNA amount	0.03	0.63	0.34	0.52	0.15	0.34	0.12	0.53

expression level in T24 cell line, but not in LD419 cells; such an effect was not seen with StaRT PCR data. PCNA transcript levels were significantly stratified by confluence in the T24 cells by both assays, but this effect is more markedly seen with StaRT PCR (Table 4). Both assays confirmed that differences in the starting RNA amount did not alter the quantification of transcripts relative to each other.

Discussion

This study confirms that StaRT PCR is a reliable and sensitive technique that can be applied to medium-high throughput quantitative transcript measurement for biological and clinical samples. We first verified that the endogenous target and competitor were amplified in a linear fashion throughout the StaRT PCR amplification cycle. Further, the CV between the calculated transcript numbers of the endogenous target was fairly low over a wide range of NT/CT ratios. We demonstrated that for the same endogenous target simultaneously amplified in multiple tubes by StaRT PCR assay, the variability between transcript numbers is very low and within the range of acceptable experimental noise. Additionally, StaRT PCR is sensitive enough to detect changes as little as 7% in endogenous target amount. StaRT PCR also compared well with the TaqMan quantitative real time PCR method in transcript quantification efficacy, thereby suggesting its use as an accurate, inexpensive, hybridization-independent alternative.

Competitive RT-PCR has evolved as a reliable strategy for quantification of gene transcripts of interest. This method has recently been standardized by mixing the defined amounts of competitors for multiple cDNAs as CT mixes in common solutions, and by generating those mixes at competitor concentrations ranging from 10^{-11}M to 10^{-16}M, covering a whole dynamic range of 6 logs of gene expression [2]. Standardized competitive RT-PCR (StaRT PCR) technique thus represents the first approach capable of studying the stoichiometric relationship between the abundance of multiple transcripts within the same sample. StaRT PCR provides an inexpensive platform for tran-

script quantification, which facilitates comparison of data generated independently in different experiments, and even in different labs [5]. Previous work with StaRT PCR has showed sufficient evidence to establish that the native as well as competitive templates amplify with the same efficiency. Since the efficiency of amplification for the native target and its competitor are identical, the ratio of their amplification products is expected to be constant and will reflect the initial transcript amounts [2]. We first verified whether endogenous target and competitor amplified at the same rate by following the NT/CT ratio from cycle 23 to 35. Further, we verified the fidelity of this amplification for different starting ratios of NT/CT over a 100-fold difference in the NT/CT ratio (0.1:1 to 10:1). When the endogenous template and its competitor existed at the same concentration (1:1) the CV across cycles was seen to be less than 4%. In addition, the CV between the calculated ACTB transcript numbers was fairly low over a wide range of NT/CT ratios. This is of interest since transcript quantification using this system is invariably performed maintaining the NT/CT ratios as close to 1:1 as possible. RB1, E2F1, PCNA and CDKN2A were also tested for linearity of amplification and yielded results similar to ACTB (CV <8 in all cases, data not shown), thus validating the potential of this technology to perform quantitative transcript measurement for any selected panel of genes. However, the CV could not be calculated for CDKN2A, where the low initial transcript number precluded the possibility of measuring PCR products until cycle 32. Since StaRT PCR is a medium-high throughput approach, transcript quantification will have to be carried out in multiple tubes. Thus, the reaction kinetics in multiple tubes must be comparable for reliable transcript measurements obtained at the end of Start PCR amplification. We have shown here that the inter-tube variability between the rates of amplification is very low and within the range of acceptable experimental noise, increasing the confidence in absolute quantification of gene transcripts by using this assay. This observation augurs well for the use of StaRT PCR to perform medium-high throughput transcript quantification.

Since many crucial cellular functions are regulated by only minute changes in expression of certain genes, the effectiveness of any transcript quantification technology largely depends on its ability to detect such small variations in transcript expression (i. e., its sensitivity). As a 'proof of principle', we have shown that StaRT PCR is capable of detecting as small as 7% change in endogenous ACTB transcripts. Given the fact that transcript quantification technology like cDNA microarray can detect, at best, only a 40% change in transcript amount [6], the ability of StaRT PCR is particularly striking. By virtue of its sensitivity in quantitative transcript measurement, StaRT PCR technology has the potential to find application in a clinical setting to improve diagnostic sensitivity and specificity.

Comparison of StaRT PCR with the established real time PCR assay for quantifying gene expression was carried out to explore its feasibility as an inexpensive, standardized, and equally sensitive alternative to the latter. The two methods cannot be compared directly since they are based on two different platforms. StaRT PCR relies on competition between endogenous and synthetic templates, while the TaqMan real time PCR is based on specificity of probe-amplicon hybridization where the fluorescent signal reports quantitatively on formation of every new copy of the template. Therefore, Spearman rank correlation test was used to compare the transcript measurement by the two methods, and the transcript estimation appears to be concordant for each of the four genes.

Conclusion

In summary, StaRT PCR is a reliable and sensitive technique that can be applied to medium-high throughput quantitative transcript measurement for biological and clinical samples. Further, it correlates well with real time PCR for its quantitative and discriminatory ability. Though StaRT PCR is relatively more labor intensive, the presence of internal standards in each PCR reaction confers multiple advantages over real time RT-PCR.First, the presence of competitive templates in each reaction mix serves as the ideal internal standard to control for reaction failure. Second, essentially similar reaction kinetics for both native and competitive templates circumvents the problems encountered in probe-based assays due to different hybridization efficiencies. Third, since transcript measurements by StaRT PCR are numerical and standardized, data generated in different labs can easily be compared and they may be entered into a common gene expression databank. Fourth, for any given cDNA sample, correlation of transcript quantification is possible, facilitating the generation of a molecular signature. In addition, owing to lack of need for fluorescent label, it is much less expensive making it more cost effective.

We have now extended the application of StaRT PCR to simultaneously quantify as many as 72 genes in 8 bladder cancer cell lines, as well as 100 frozen bladder cancer samples (manuscripts in preparation). Generation of molecular signatures unique to individual tumors and profiles for genes across tumor samples will provide an additional prognostic tool and help in identifying novel therapeutic targets. Moreover, StaRT PCR offers the possibility of multiplex amplification of several genes in the same reaction mix. Efforts in this direction have already yielded promising results [7]. Further, the StaRT PCR technology can be combined with micro-fluidic electrophoresis and robotic liquid handling, and such application has recently

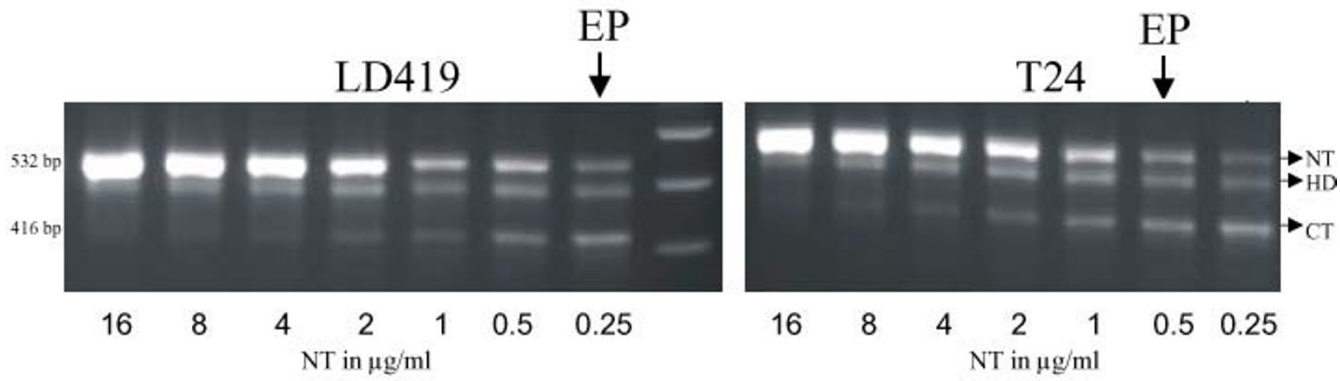

Figure 3
Determination of an Equivalence Point (EP) in bladder cell lines LD419 and T24: Equivalence point for any sample is defined as the concentration of cDNA, which yields a visually equivalent band intensity between the NT & CT (1:1 ratio) for ACTB. cDNA dilution approximating the equivalence point is chosen for further quantification experiments. Decreasing concentrations of cDNA (NT) were separately added to a constant volume of CT mix (containing ACTB competitor at 10^{-12}M), and the mixtures were amplified using ACTB primers for 35 cycles. The PCR products were electrophoresed and the 'equivalence points' were visually assigned for LD419 and T24 cDNA (arrows).

allowed expression measurement in a semi-automated fashion [7].

Materials and Methods

Cell Culture

One human bladder transitional carcinoma cell line, T24 (ATCC, Manassas, VA, USA), and a normal fibroblastic bladder primary culture cell line, LD 419 (developed and kindly provided by Dr. P. Jones, University of Southern California, USA), were grown in McCoys 5a medium (Invitrogen, Carlsbad, CA, USA), with 10% FBS (CRL core facility University of Southern California, USA). Cells were harvested at approximately 60% and 100% confluence, and after cell count, each cell pellet was equally distributed into three aliquots of 10^7 cells. Thus, a total of 12 aliquots were prepared for RNA extraction from the two cell types.

RNA Extraction and cDNA synthesis

RNA was extracted using the RNeasy midi kit from Qiagen (Valencia, CA, USA) following the vendor's instructions. For each of the two culture conditions, reverse transcription was performed starting with 5 µg, 10 µg and 15 µg of total RNA in triplicates, yielding a total of 36 samples for the two cell lines. In each case, the cDNA preparation was performed in a 50 µl reaction volume containing 200 U Moloney Murine Leukemia Virus (MMLV) reverse transcriptase, 2 mM dNTPs, 300 ng of random hexamers and 40 U of recombinant ribonuclease inhibitor in reverse transcription buffer (50 mMTris-HCl, 75 mM KCl, 3 mM $MgCl_2$ and 2 mM dithioerythreitol). All reagents were obtained from Invitrogen. The reaction was carried out for 1 h at 45°C, followed by reverse transcriptase inactivation for 5 min. at 95°C.

StaRT PCR

A ready-to-use master mixture (MM containing *Taq* DNA polymerase 1.25 units, dNTPs 0.2 mM each, reaction buffer, 1.5 mM $MgCl_2$, gel loading dye and precipitant) (1.1 X ReddyMix, Cat # AB-0575 LD/C, Abgene, Epsom U.K.) was used to reduce risk of contamination and pipetting errors. StaRT PCR was performed using internal standard competitive template (CT) mixtures (A-F) from Gene Express, Inc. (Toledo, OH). While each of the six mixtures (A-F) contained internal standard CTs for 381 "target" genes, our pilot study targeted RB, E2F1, CDKN2A and PCNA genes. The concentration of β-actin (ACTB) internal standard CT was 10^{-12} M (6×10^5 molecules/l) in each mix, while the concentration of the internal standards for the "target" genes varied serially 10 fold

Table 5: Primers and probes used for real-time PCR analysis for 5 target genes

Gene	Forward Primer	Reverse Primer	Probe
ACTB	TGAGCGCGGCTACAGCTT	CCTTAATGTCACACACGATT	6FAM5'-ACCACCACGGCCGAGCGG-3'TAMRA
PCNA	CTGCAGAGCATGGACTCGTC	GTAGGTGTCGAAGCCCTCAGA	6FAM5'-ACGTCTCTTTGGTGCAGCTCACCCTG-3'TAMRA
CDKN2A	CATAGATGCCGCGGAAGGT	CCGGAGGTTTCTCAGAGCCT	6FAM5'-CCTCAGACATCCCCGATTGAAAGAACC-3'TAMRA
E2F1	GAACTGAGGCCTGGGTGATTT	CCACCCATGGCTGTCAGTC	6FAM5'-TCTGTCTCCCTCCCTCACTTTCCCAATAA-3'TAMRA
RB1	TTCCAGAAAATAAATCAGATGGTATGTAA	CAGTGGTTTAGGAGGGTTGCTT	6FAM5'-CAGCACTTCTTTTGAGCACACGGTCG-3'TAMRA

Primers used for StaRT PCR analysis for 5 target genes

Gene	Forward primer	Reverse Primer for NT	Reverse Primer for CT
ACTB	GATTCCTATGTGGGCGACGAG	CCATCTCTTGCTCGAAGTCC	CCATCTCTTGCTCGAAGTCCCCCATCTCTTGCTCGAAGTC CGCCAGCCAGGTCCAGACGCA
CDKN2A	GGAGCCCAACTGCGCCGACC	CCTTCGGTGACTGATGATCTAA	CCTTCGGTGACTGATGATCTAAGGAGCCCAACTGCGCC GACCCCCGTGGACCTGGCTGAG
E2F1	TGATACCCCAACTCCCTCTA	AAAGCAGGAGGGAACAGAGC	AAAGCAGGAGGGAACAGAGCAAAGCAGGAGGGAACAG AGCACTGCAGGGACCACAGG
PCNA	GCTCCAGCGGTGTAAACCTGCA	CGTGCAAATTCACCAGAAGGCA	CGTGCAAATTCACCAGAAGGCACGTGCAAATTCACCAG AAGGCATCAACTTCATTTCATAGTCTGA
RB1	TTTCAGAAGGTCTGCCAACACCAA	GTGTCCACCAAGGTCCTGAGATCC	GTGTCCACCAAGGTCCTGAGATCCGTGTCCACCAAGGT CCTGAGATCCCATTTCTGCCAGTTTCTGCTGAAA

from 10^{-11}M in Mix A through 10^{-16} M in Mix F. This enabled the quantification of the target transcripts across 6 logs of concentrations. Since every CT mix contained ACTB CT molecules at 10^{-12} M concentration, 1 μl was equivalent to 6×10^5 molecules. Normalization of the test sample was performed to identify the concentration of the test cDNA sample where the ACTB transcripts existed at 6×10^5 molecules. To achieve this, serial dilutions of a cDNA sample were mixed with a constant amount (1 μl) of ACTB competitor contained in one of the CT mixes. A typical 50 μl PCR reaction contained 1 μl of CT mix, 1 μl of test cDNA sample containing native template (NT), 0.5 μl of each of the ACTB primers and 47 μl of MM. Following gel electrophoresis of the PCR products, the concentration of the test cDNA sample at which the band intensities of the NT was visually equivalent to that of the CT was designated as the 'equivalence point'. For any test sample, the cDNA concentration close to the equivalence point was chosen to proceed for further quantification experiments (figure 3). Thus, for each sample a master mix containing the CT mix and the cDNA sufficient for expression measurements of the five genes (cell cycle specific genes, and ACTB) were prepared and aliquoted into five tubes containing the respective primers. The procedure was repeated for each of the CT mixes. Normalization and quantification reactions were carried out in a PTC-100 thermal cycler (MJ research, Waltham MA, USA) for 35 cycles, with the following thermal profile: initial denaturation step-95° C, 5 minutes, followed by 35 cycles of denaturation at 94° C, 1 minute annealing at 58° C, 1 minute, extension at 72° C, 1 minute. A final 10-minute extension at 72°C was followed by a 4°C soak until the samples were used for gel electrophoretic analysis. The primers and the internal sequences used to generate the CT are listed in table 5.

Data Analysis and quantitation

Following PCR, amplification products were electrophoresed in a 3% SeaKem agarose gel (BMA, Rockland, ME, USA), using a 104 lane high-throughput electrophoresis system (CBS, Del Mar, CA, USA, cat# SGU 2640T-02), and stained with 0.05 μg/ml ethidium bromide. A computer-integrated system comprising Fluor-S-MultiImager Hardware and Quantity One software (Bio-Rad, Hercules, CA, USA) was used for digital acquisition of gel images and for quantification of the band fluorescence intensities.

In all cases, band intensity was expressed as volume under the curve: pixel intensity × mm^2. Determination of the number of molecules present in each band has been described [2]. NT pixel intensity was corrected taking into account the size difference between NT and CT (corrected NT pixel intensity=observed NT pixel intensity × NT size / CT size). A third band corresponding to the hetero-duplex was often observed between NT and CT. Since hetero-duplex formation disturbs the final homo-duplex product ratios, it was essential that hetero-duplexes were identified, accurately quantified and their contribution to final product ratios included in the analysis [3]. Hence, we attributed half the pixel intensity of each hetero-duplex to the corrected NT pixel intensity and the other half to the CT pixel intensity. The initial number of cDNA (NT) molecules was then calculated as [(corr. NT pixel intensity /

CT pixel intensity) × CT molecules]. For every target gene, ACTB transcripts were also measured for standardization of the results, and for each gene the number of transcripts was expressed as number of copies per 10^6 ACTB molecules.

Testing Linearity of StaRT PCR amplification

The Start PCR system uses end point analysis to quantify the initial number of transcripts of a particular gene in a given sample. As described above, the relative band intensities of the NT, CT and HD following StaRT PCR amplification and gel electrophoretic analysis are assessed and used to calculate the initial transcript number in the NT. Therefore, we investigated:

a) The linearity of Standardized Competitive RT-PCR (StaRT PCR) amplification system

b) Coefficients of Variation (CV) between the initial ACTB transcript numbers in the NT for each of the designed NT/CT ratios.

The linearity of Start PCR amplification was tested as follows: LD419 cell line was used to quantify the native (endogenous) ACTB transcripts by measuring the NT/CT ratios at cycle numbers 23, 26, 29, 32 & 35. LD419 cDNA was mixed with the CT to arrive at NT/CT ratios of 0.1, 0.5, 1, 5, and 10. Independently, each of these 5 initial NT/CT ratios were used to set up separate reactions, which were then subjected to PCR amplification and terminated at cycles 23, 26, 29, 32, 35. All experiments were performed in triplicate. The mean observed NT/CT ratios (following quantification using BioRad Quantity One software) at each end point were plotted against the cycle numbers at which the reactions were terminated. Similar analysis was performed for each of the other four genes, RB1, E2F1, CDKN2A, and PCNA, at the same NT/CT ratios, following empirical determination of the appropriate CT mix (from among A-F) for respective genes.

Further, using replicate experiments, coefficients of variation (CV) were calculated between the initial ACTB transcript numbers in the NT obtained based on the observed NT/CT ratios at the end of StaRT PCR amplification for each of the designed NT/CT ratios.

Testing Inter-tube variability in StaRT PCR amplification

Since expression profiling and signature generation studies for clinical cDNA samples using gene panels would be done using separate reaction tubes, we compared the variability in transcript quantification between multiple tubes subjected simultaneously to the StaRT PCR amplification system. In this experiment to test the inter sample variability, we quantified the number of native ACTB transcripts for the in LD419 cell line. We used NT/CT ratio of 1:1 based on our results of the CV calculation described above. Equal inputs of cDNA for ACTB transcripts were amplified in multiple tubes simultaneously and the PCR reactions terminated at cycles 20, 23, 26, 29, 32 & 35. Experiments were performed in triplicate. The ACTB transcripts in each of the tubes were quantified at the designated end points and the variability between the values obtained from the multiple tubes compared.

Determination of Sensitivity of StaRT PCR in detecting small changes in transcript amount

The effectiveness of any quantitative technology for gene expression depends to a large measure on its sensitivity to detect small differences in values for individual samples, since many crucial cellular functions are regulated by minute changes in gene expression. NT/CT ratios were therefore designed to test the ability of StaRT PCR to detect minute changes in transcript quantity. LD419 and T24 cDNAs were normalized using ACTB primers (figure 3). In each case, NT volumes decreasing successively by 0.4 μL were mixed with a constant volume of CT (4 μL of 10^{-12}M ACTB) to obtain NT/CT dilutions over a range of 1.5:1 to 0.5:1. This allowed us to test the sensitivity of StaRT PCR over a demanding range of expression changes from 6%-15%. The starting cDNA concentrations used for this experiment were visually determined to be different for T24 and LD419; cDNA volume for T24 chosen was the one that yielded the equivalence point, while that for LD419 was approximately 1.5 times the one required for equivalence point. These different cDNA volumes were deliberately chosen to examine the consistency of transcript quantification over equal successive decrease in ACTB transcript number for the two cell lines (6%-15%), in spite of the differences in starting transcript numbers. These reaction mixes were then subjected to StaRT PCR amplification using ACTB primers and the corresponding number of ACTB transcripts in the NT was calculated. All experiments were performed in triplicate. The mean absolute numbers of ACTB transcripts quantified from each NT/CT titration were plotted against the NT/CT ratio.

Comparison of StaRT PCR with real time PCR

In order to compare the transcript quantification by StaRT PCR and real time PCR, T24 and LD419 cells were grown to two different confluence conditions, 60% and 100%, and harvested to extract total RNA. From each of these total RNA extracts, RNA amounts equivalent to 5, 10 & 15 μg were reverse transcribed in triplicates to obtain a total of 36 cDNA preparations. ACTB, RB1, E2F1, CDKN2A and PCNA transcripts were subsequently quantified from each cDNA preparation using StaRT PCR and real time PCR.

Real time PCR primers and probes design

The quantification of mRNA was carried out using a real time fluorescence detection method essentially as

described. Briefly, each of the cDNAs of interest was PCR-amplified using unlabeled primers in presence of a gene specific oligonucleotide probe with a 5' fluorescent reporter dye (6FAM) and a 3' quencher dye (TAMRA). During each primer extension step, the 5' to 3' exonuclease activity of Taq DNA polymerase cleaves the annealed probe and releases the fluorescent reporter away from the quencher moiety, resulting in a fluorescent signal, which was then detected using a suitable detector system (ABI Prism 7700 Sequence Detection System, (Perkin-Elmer, Foster City, CA). Initial template concentration was derived from the cycle number at which the fluorescent signal crosses a threshold in the exponential phase of the PCR reaction. Relative gene expression was determined based on the threshold cycles (Ct values) of the gene of interest (RB1, CDKN2A (p16), PCNA and E2F1) and of the internal reference gene (ACTB). The PCR amplification was performed using a 96-well optical tray and caps with a final reaction mixture of 30 ml which consisted of 300 nM of each primer, 100 nM probe, 0.5 U Ampli-Taq Gold, 240 mM each of dATP, dCTP, dGTP, TTP, 3.5 mM MgCl2, and 1× TaqMan Buffer A containing a reference dye at the following conditions: 95°C for 10 min, followed by 40 cycles at 95°C for 15 s and 60°C for 1 min. The primer and probe sequences are listed in table 5.

Statistical Analysis

All statistical calculations were performed using SAS software. Relevant tests are described under the respective sections below.

Authors' contribution

VP and BG were responsible for carrying out all experiments, transcript quantification, data analysis and manuscript preparation. SB assisted with maintaining cell cultures, RNA preparation and manuscript editing. JW provided cloned competitive templates, which served as internal standards, and provided inputs on the StaRT-PCR assay quality control. PWL directed the Taqman Real-time RT-PCR assays. JC and SG performed the statistical analysis. RJC and RHD were responsible for designing the experiments, overall organization of the research project and manuscript preparation. All authors read and approved the final manuscript.

Abbreviations

RT-PCR: Reverse Transcriptase – Polymerase Chain Reaction

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