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Multiplex standardized RT-PCR for expression analysis of many genes in small samples

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Abstract

Standardized RT-PCR (StaRT-PCR) enables numerical quantification as well as intra- and inter-laboratory comparison of gene expression. Multiplex StaRT-PCR, using two rounds of amplification, was conducted on Stratagene Universal Reference RNA. In the first round, cDNA, competitive template (CT) mix, and primers for up to 96 genes were amplified for varying numbers of cycles. Next, products from round one were diluted, combined with primers for one gene, and amplified for an additional 35 cycles. No additional cDNA or CT mix was added. Expression values obtained by uniplex and multiplex StaRT-PCRs were highly correlated ($R = 0.993$, $p < 0.001$). Products from round one could be diluted as much as 100,000-fold and still be quantified following round two amplification. Thus, using multiplex StaRT-PCR, 96 genes were measured in the same amount of cDNA typically used to measure one gene with uniplex StaRT-PCR. Multiplex StaRT-PCR was also used to measure 18 genes in the fine needle biopsy of a primary lung carcinoma.

Keywords: Standardized; Multiplex; Quantitative; RT-PCR; mRNA; Gene expression; Competitive template; FNA

It is increasingly recognized that, in order for gene expression measurements to be useful in a clinical setting, data must be both quantitative and standardized [1–3]. Recently published studies using Standardized RT (StaRT)-PCR have identified patterns of gene expression associated with lung cancer [4–6], pulmonary sarcoidosis [7], cystic fibrosis [2], and chemoresistance in childhood leukemias [8,9]. The StaRT-PCR method used in these studies provides quantitative end-point gene expression measurements because internal standard competative templates (CTs) are included in each reaction [1]. StaRT-PCR uses cDNA from as few as 100–1000 cells which enables analysis of very small specimens [1]. However, there are multiple reasons to further reduce the amounts of cDNA and reagents required in each PCR. First, the throughput capacity for gene expression measurement is increasing with implementation of capillary electrophoresis (CE) devices [10] and robotic liquid handling systems. Second, the size of biopsies obtained in many clinical situations is decreasing as cytologic methods improve and economic pressure to reduce costs increases. For example, samples of suspected cancerous lesions in the lung, breast, prostate, thyroid, and pancreas, are commonly obtained by fine needle aspirate (FNA) biopsy. There is also a need to evaluate expression patterns in samples from anatomically small, but functionally important tissues of the brain, developing embryo, and animal models, including laser capture micro-dissected samples and flow-sorted cell populations. Third, it may be necessary to measure 50–100 genes in these small samples to fully characterize a phenotype [11]. It is important though to ensure that enough cDNA is used to detect rare transcripts and that the relationship of one transcript to another is not altered by the detection method. One way to achieve these goals would be to multiplex amplify many genes in the presence of a known amount of CT for each gene. The presence of CTs in

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[1] These authors each have a significant financial interest in Gene Express, Inc., the parent company of Gene Express National Enterprises, Inc., that produces and markets protocols and reagents for StaRT-PCR.

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multiplex reactions controls for unpredictable inter-sample variation in the efficiency of amplification during the course of PCR caused by reagent consumption and/or product inhibition. In addition, CT internal standards control for preferential amplification of one transcript over another due to differences in priming efficiencies.

We have previously reported StaRT-PCR amplification of native templates (NT) from cDNA and corresponding CTs for two genes in a single PCR [1] but previous efforts with more than two genes in a single reaction did not result in quantifiable bands. Here, quantifiable PCR products for many genes were obtained using a variation of multiplex StaRT-PCR in which the cDNA and CTs were PCR amplified in two 35-cycle rounds. In the first round, primers for multiple genes were present along with a mix of corresponding CTs and cDNA. In round two, aliquots of the round one amplification products were further amplified with primers for single genes in separate reactions.

Materials and methods

Reagents

PCR buffer (10X) for the Rapidcycler (500 mM Tris, pH 8.3, 2.5 μg/μl BSA, 30 mM $MgCl_2$) was obtained from Idaho Technology (Idaho Falls, Idaho). Thermo 10X buffer (500 mM KCl, 100 mM Tris–HCl, pH 9.0, 1.0% Triton X-100), *Taq* polymerase (5 U/μl), oligo dT primers, RNasin (25 U/μl), pGEM size marker, and dNTPs were obtained from Promega (Madison, WI). M-MLV reverse transcriptase (200 U/μl) and 5X first strand buffer (250 mM Tris–HCl, pH 8.3, 375 mM KCl, 15 mM $MgCl_2$, 50 mM DTT) were obtained from GibcoBRL (Gaithersburg, MD). NuSieve and SeaKem LE agarose were obtained from FMC BioProducts (Rockland, ME). TriReagent was obtained from Molecular Research Center (Cincinnati, OH). RNase-free water was obtained from Research Genetics (Huntsville, AL). DNA 7500 Assay kit containing dye, matrix, and standards was obtained from Agilent Technologies (Palo Alto, CA). The lung adenocarcinoma cell line, A549, was purchased from American Type Culture Collection (Rockville, MD). RPMI-1640 cell culture medium was obtained from Sigma (St. Louis, MO). Universal Human Reference RNA was obtained from Stratagene (La Jolla, CA). GENE system 1 and system 1a gene expression kits were provided by Gene Express National Enterprises (Huntsville, AL). All other chemicals and reagents were of molecular biology grade.

Bronchogenic carcinoma sample acquisition

A fine needle aspirate (FNA) of a primary non-small cell lung cancer was obtained from a 67-year-old male at the Medical College of Ohio. Cells not placed immediately on slides for diagnostic purposes were stored in Preservcyt Solution (CYTYC, Boxborough, MA). After a final diagnosis was made through cytological assessment, the remaining cells were pelleted in our research laboratory and RNA was extracted as described below. Informed consent was obtained from the patient according to NIH and institutional guidelines.

RNA extraction and reverse transcription

Total RNA from the FNA was extracted according to the TriReagent Manufacturer Protocol [12]. Universal Human Reference RNA was precipitated according to manufacturer's protocol. Approximately 1 μg total RNA was reverse transcribed using M-MLV reverse transcriptase and an oligo dT primer as previously reported [13].

Uniplex StaRT-PCR

Standardized RT-PCR was performed using previously published protocols [1,14] with GENE system I or system Ia gene expression kit (Gene Express National Enterprises). Each kit contained six mixtures of the CTs and corresponding primers. Each of the six mixes contained CTs for the same genes but the concentration of the "target gene" CTs in each mix varied compared to the concentration of the "housekeeping gene" CTs. Briefly, a master mixture containing buffer, $MgCl_2$, dNTPs, cDNA, CT mixture from GENE system 1 or system 1a kit and *Taq* polymerase was prepared and aliquotted into tubes containing gene-specific primers and cycled either in a Rapidcycler (Idaho Technology) or Primus HT Multiblock thermal cycler (MWG-BIOTECH, High Point, NC) for 35 cycles. In each protocol the denaturation temperature was 94 °C, the annealing temperature was 58 °C, and the elongation temperature was 72 °C. For the Rapidcyler, the denaturation time was 5 s, the annealing time was 10 s, the elongation time was 15 s, and the slope was 9.9. For the Primus HT Multiblock, the denaturation, annealing, and elongation times were each 1 min, the lid temperature was 110 °C, and the lid pressure was 150 Newtons. PCR products were evaluated on an agarose gel or in the Agilent 2100 Bioanalyzer (Agilent Technologies) as described below.

Multiplex StaRT-PCR amplification of nine genes

Different initial multiplex StaRT-PCR conditions were used to test and optimize the method. Each multiplex StaRT-PCR was amplified in two rounds. In the first round, one reaction was set up containing buffer, $MgCl_2$, dNTPs, a previously prepared mixture of cDNA and CT mixture (cDNA from A549 p85 and one of the CT mixes from GENE system 1a), *Taq* polymerase, and primer pairs for nine genes. This reaction was cycled for 5, 8, 10, or 35 cycles. The concentration of each primer in the primer mix was 0.05 μg/μl. Following amplification, this PCR product was diluted with water for use as a template in round two.

In round two, a master mixture containing buffer, $MgCl_2$, *Taq* polymerase, and a primer pair specific for one gene was aliquotted into tubes containing 1 μl of each of the following dilutions of PCR product from the first round: undiluted, 1/5, 1/10, 1/50, 1/100, 1/1000, 1/10,000, 1/100,000, and 1/1,000,000. These reactions were cycled 35 times and then electrophoretically separated and detected on an agarose gel or in the Agilent 2100 Bioanalyzer as described below. Primer pairs used in this round were selected from among the primer pairs used in round one. No additional cDNA or CT mix was added into the PCR in round two.

For control uniplex StaRT-PCR, the mixture of cDNA and CT mixture prepared for use in round one of the nine gene multiplex reactions were serially diluted prior to amplification: undiluted, 1/5, 1/10, 1/50, 1/100, 1/1000, 1/10,000. A 1 μl aliquot of each dilution was combined with an aliquot of a master mixture containing buffer, $MgCl_2$, *Taq* polymerase, and a primer pair specific for one gene (0.05 μg/μl of each primer). These reactions were amplified with only one round of 35 cycles according to previously described, standard conditions (see above and Refs. [1,14]).

Multiplex StaRT-PCR amplification of 96 genes

In an effort to increase the number of genes measured by multiplex StaRT-PCR, additional experiments were conducted. Samples of cDNA derived from Stratagene Universal Human Reference RNA and CT mixes from GENE system 1 (which contain CTs for 96 genes) were used in these experiments. A solution containing primers for each of the 96 genes represented by CTs in GENE system 1 was included in the first round reactions. It was necessary to dilute this 96 gene primer mix

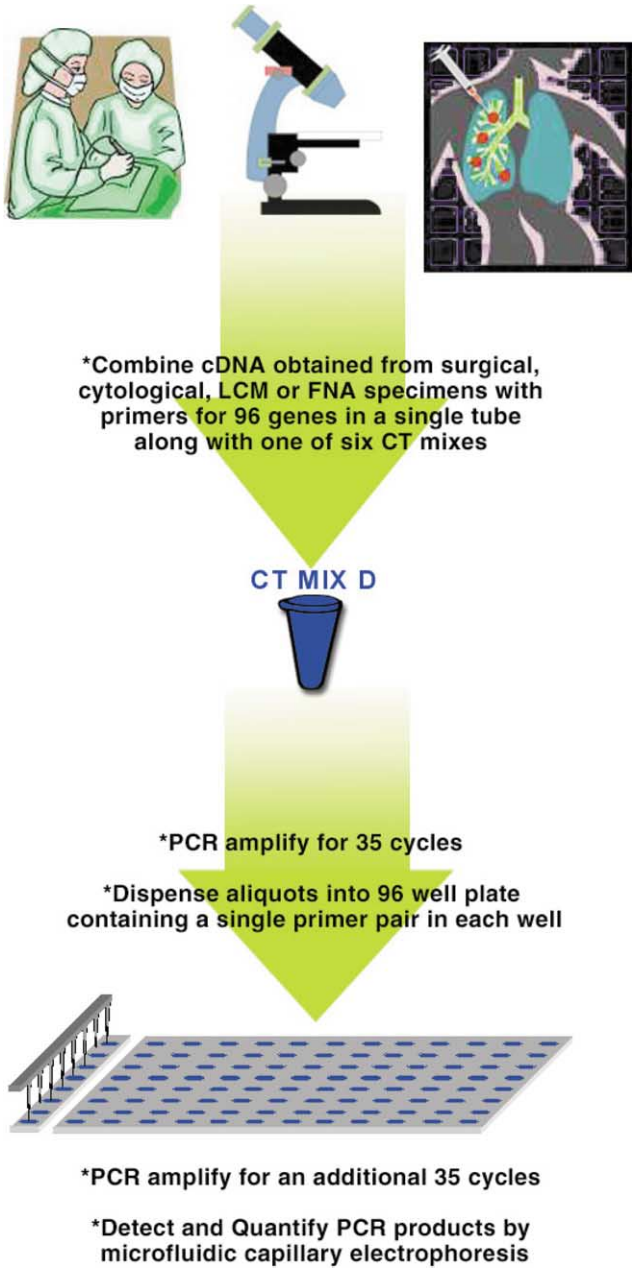

Fig. 1. Schematic representation of multiplex StaRT-PCR. A small amount of cDNA from a patient sample is PCR amplified with primer pairs for 96 genes and one CT mix containing CTs for the same 96 genes. In the example shown here, CT mix D is used. Following this multiplex reaction, PCR product is aliquotted into each well of a 96 well plate. Each well of the plate contains a single primer pair for one of the original 96 genes previously amplified in multiplex. An additional PCR amplification is done in the 96 well plate and PCR products are detected and quantified using CE. This process can be repeated with a different CT mix for any gene that the NT/CT ratio is out of balance. Conversely, tubes containing cDNA and each of the six CT mixes can be amplified simultaneously.

so that the concentration of each primer was 0.005 μg/μl. Every round one reaction was cycled 35 times. Round one PCR products were then diluted 100-fold (1 μl of round one product into 99 μl water). One microliter of diluted round one PCR product was used in each round two reaction along with primers for a single gene selected from among those amplified in round one and cycled 35 times (Fig. 1).

Control uniplex reactions were conducted using samples of cDNA derived from Stratagene Universal Human Reference RNA and CT mixes from GENE system 1 as described previously [1,14] and above. For these experiments, no dilution of the cDNA or CT mix was done prior to amplification.

Multiplex StaRT-PCR assessment of primary bronchogenic carcinoma

Following the optimization of multiplex StaRT-PCR described above, the primary bronchogenic carcinoma obtained by FNA was assessed. CT mixes from GENE system 1 were combined with cDNA from the FNA sample and amplified in multiplex in round one. Eighteen putative chemoresistance genes were amplified from round one PCR product in round two.

Electrophoresis and quantitation

Agarose gel electrophoresis. Following amplification, PCR products were loaded directly into 4% agarose gels (3:1 NuSieve: SeaKem) containing 0.5 μg/ml ethidium bromide. Gels were electrophoresed for approximately one hour at 225 V. Electrophoresis buffer was cooled and recirculated during electrophoresis. Gels were visualized with a Foto/Eclipse image analysis system (Fotodyne, Hartland, WI). Digital images were saved on a Power Mac 7100/66 computer and Collage software (Fotodyne) was employed for densitometric analysis [1,14].

Agilent 2100 bioanalyzer microcapillary electrophoresis. Following amplification, 1 μl of each 10 μl PCR was loaded into a well of a chip prepared according to manufacturer's protocol [15] for the DNA 7500 Assay. Briefly, 9 μl gel-dye matrix was loaded into the chip in one well and the chips were pressurized for 30 s. Two additional wells were filled with gel-dye matrix and the remaining wells each were loaded with 5 μl molecular weight marker. One microliter of DNA ladder was loaded into a ladder well and 1 μl PCR product was loaded into each sample well. The chip was vortexed and placed into the Agilent 2100 Bioanalyzer. The DNA 7500 Assay program was run which applies a current sequentially to each sample to separate products. DNA was detected by fluorescence of the intercalating dye in the gel-dye matrix.

Quantification of gene expression. Quantification of gene expression was determined as previously reported [10]. Band intensities were used for quantification when agarose gel electrophoresis was employed and the area under the curve was used when PCR products were detected with the Agilent 2100 Bioanalyzer. First, the NT/CT ratio of a housekeeping gene, β-actin, and the NT/CT ratios for each target gene were calculated. Because the initial concentration of CT added into the PCR was known, the initial NT concentration could be determined. Since each NT/CT ratio was based on intercalater dye staining of the PCR products and this staining is affected both by the number of molecules present and the length of the molecules in base pairs, NTs were arbitrarily corrected to the size of the CT product prior to taking the NT/CT ratio. Heterodimers (HD), when measurable, were corrected to the size of the CT and divided by two. One half of the HD value was added to the NT and one half was added to the CT prior to taking the NT/CT ratio since one strand of the HD comes from the NT and the other comes from the CT. Second, the calculated number of target gene NT molecules was divided by the calculated number of β-actin NT molecules to correct for loading differences.

For multiplex StaRT-PCR, target genes detected under each condition (varying dilutions and/or round one cycle number) were measured against β-actin detected under the same condition. For example, round one of the nine gene multiplex reaction contained primers for nine genes including both β-actin and c-*myc*. A 1/100,000 dilution of the PCR from round one was made and used in round two. An aliquot

Table 1
Effect of cycle number on multiplex StaRT-PCR

Gene	Round one cycles	Round two cycles	Maximum dilution[a]	Gene expression value[b]
β-actin	35	0	1/10	NA[c]
	5	35	1/100	NA
	8	35	1/100	NA
	10	35	1/1000	NA
	35	35	1/100,000	NA
c-*myc*	35	0	1/1000	9.0E + 04
	5	35	1/1000	8.2E + 04
	8	35	1/10,000	7.0E + 04
	10	35	1/10,000	3.3E + 04
	35	35	1/1,000,000	3.4E + 04
Catalase	35	0	1/1000	1.4E + 04
	5	35	1/1000	3.8E + 04
	8	35	1/10,000	8.8E + 03
	10	35	1/10,000	1.2E + 04
	35	35	1/1,000,000	9.3E + 03

[a] Maximum dilution at which quantifiable PCR products could be detected. For uniplex reactions (round two cycles = 0), the dilution shown is of the starting mixture of cDNA and CT mix. For multiplex reactions, the dilutions shown are of round one products (e.g. 1/100 = 1 μl of the 10 μl round one product +9 μl water).

[b] Gene expression values are reported as molecules per 10^6 β-actin molecules.

[c] NA = Not applicable.

of this dilution was used in round two to amplify both β-actin and c-*myc*. Under these conditions, c-*myc* was measured as 3.40×10^4 molecules/10^6 β-actin molecules when cycled 35 times in round one and 35 times in round two (Table 1).

Statistical analysis

All statistical analyses were conducted using SPSS version 9.0 for Windows. A two-tailed Pearson's Correlation test was conducted on logarithmically transformed data to compare gene expression values obtained by uniplex StaRT-PCR with those obtained by multiplex StaRT-PCR. The correlation was considered statistically significant if the p value was less than 0.05.

Results

Multiplex StaRT-PCR amplification of nine genes

After 35 cycles of amplification in round one with primer pairs for nine genes, aliquots of the PCR products were diluted and amplified with primers for one of the nine genes. Bright, distinct bands were observed for each gene (Fig. 2). Thus, the same amount of cDNA and CT mix that is used in a typical uniplex StaRT-PCR to measure one gene in one round of amplification was used to obtain nine gene expression measurements in multiplex StaRT-PCR.

Further, the round one PCR product could be diluted as much as 1,000,000-fold for catalase or c-*myc* (100,000-fold for β-actin) and still be quantified following amplification with primer pairs for one gene in round two (Table 1, Fig. 2). In contrast, when the cDNA and CT mix used in round one was diluted more than 1000-fold prior to amplification (100-fold or more for β-actin) and then amplified with a single primer pair for any one of these genes in a single round of 35 cycles, no detectable product was observed.

Increasing the number of cycles used in round one increased the amount of the PCR product that could be diluted prior to round two and still be detectable after round two amplification. Therefore, more gene expression measurements can be made on a sample when it is amplified using multiplex StaRT-PCR with 35 cycles used in each round than when fewer cycles (5, 8, and 10 cycles) are used in round one or when uniplex StaRT-PCR is used. Details for each gene and each condition are shown in Table 1. Representative gels of control uniplex and multiplex reactions are shown in Fig. 2.

Multiplex StaRT-PCR amplification of ninety-six genes

Gene expression values obtained by uniplex and 96 gene multiplex StaRT-PCR of the cDNA derived from Stratagene Universal Human Reference RNA are shown in Table 2. Although 96 primer pairs were included in the multiplex reactions, gene expression values for only 93 genes are reported because (1) each gene expression value is reported as molecules of target gene/10^6 molecules of β-actin so β-actin values are not reported, (2) although two sets of reagents to measure GAPD gene expression (GAPD CT1 and GAPD CT2) are included in the GENE system 1 kit (see methods), only GAPD CT1 was measured in this sample and, (3) reagents for one gene, BAX alpha, provided in the kit did not pass quality control testing done by GENE, so this gene was not assessed in this study. Bivariate analysis of uniplex and multiplex

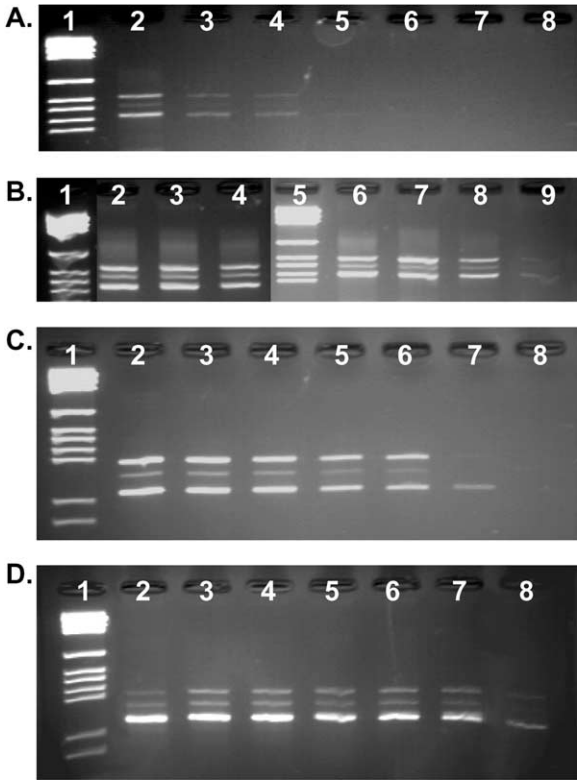

Fig. 2. Multiplex vs. uniplex StaRT-PCRs. For A–D, following amplification, StaRT-PCR products were electrophoresed on 4% agarose gels. GENE system 1a CT mix D and cDNA from A549 p85 were mixed together and amplified in uniplex and multiplex StaRT-PCRs. A: Control uniplex reaction with β-actin primers. Lane 1, pGEM size marker; lane 2, PCR contained undiluted cDNA with β-actin NT in balance with 300,000 molecules of β-actin CT; lane 3, PCRs contained 1:5 diluted cDNA/CTmix; lane 4, 1:10 diluted cDNA/CT mix; lane 5, 1:50 diluted cDNA/CT mix; lane 6, 1:100 diluted cDNA/CT mix; lane 7, 1:1000 diluted cDNA/CT mix; lane 8, 1:10,000 diluted cDNA/CT mix. B: PCR products from the second round multiplex StaRT-PCRs, each of which contained an aliquot of round one PCR product and β-actin primers. Lane 1, pGEM size marker; lane 2, 1/500th of the round one 10 μl PCR product (1 μl of a 1:50 dilution); lane 3, 1/1000th round one PCR product; lane 4, 1/10,000th round one PCR product; lane 5, pGEM size marker, lane 6, 1/10,000th round one PCR product; lane 7, 1/100,000th round one PCR product; lane 8, 1/1,000,000th round one PCR product; lane 9, 1/10,000,000th round one PCR product. C: Control reaction with catalase primers. Lane 1, pGEM size marker; lane 2, PCR contained undiluted cDNA and CT mix, equivalent to 3000 molecules of catalase CT; lane 3, 1:5 diluted cDNA/CT mix; lane 4, 1:10 diluted cDNA/CT mix; lane 5, 1:50 diluted cDNA/CT mix; lane 6, 1:100 diluted cDNA/CT mix; lane 7, 1:1000 diluted cDNA/CT mix; lane 8, 1:10,000 diluted cDNA/CT mix. D: PCR products for the second round of multiplex StaRT-PCR. Reactions included an aliquot of round one PCR product and catalase primers. Lane 1, pGEM size marker; lane 2, 1/100th of the 10 μl round one PCR product (1 μl of a 1:10 dilution); lane 3, 1/500th round one PCR product; lane 4, 1/1000th round one PCR product; lane 5, 1/10,000th round one PCR product; lane 6, 1/100,000th round one PCR product; lane 7, 1/1,000,000th round one PCR product; lane 8, 1/10,000,000th round one PCR product.

StaRT-PCR gene expression values revealed a highly significant ($p = 0.001$) positive correlation ($r = 0.993$) (Fig. 3).

Multiplex StaRT-PCR assessment of primary bronchogenic carcinoma

Eighteen genes putatively associated with carboplatin chemoresistance were assessed by multiplex StaRT-PCR in an FNA sample determined to be positive for non-small cell carcinoma by cytology. Mean expression levels ranged from none detected to 8.1×10^6 molecules/10^6 β-actin molecules with an average coefficient of variation of 0.33 (Table 3). Expression levels were considered not detectable when CT for the gene was measured in a reaction at its lowest concentration (6 molecules) and no NT for the gene could be detected.

Discussion

The use of StaRT-PCR to identify patterns of gene expression has many advantages. Because an internal standard is included in each reaction, the data are numerical and standardized and they are readily comparable between samples and laboratories [16]. The numerical format enables the combination of gene expression values into indices that better define specific phenotypes [4,5,8,9]. Compared to other methods of measuring gene expression, StaRT-PCR is rapid, inexpensive, sensitive, and highly compatible with automation. Because internal standards (CTs) for each gene are included in the reaction, data may be collected at the end point of the reaction. Real-time measurement of PCR products, which requires expensive equipment and sequence-specific fluorescent probes, is avoided. Since CTs are prepared in mixtures with varied concentrations relative to housekeeping gene CTs, there is no need for extensive titrations or the generation of standard curves. If the NT/CT ratio is outside acceptable limits for a particular gene, that gene simply can be reassessed using a different CT mix provided in the kit. Alternatively, samples may be assessed with each concentration of the CT mix simultaneously to increase throughput.

The efficiency of amplification of identical templates varies in different samples [17]. Using StaRT-PCR, direct comparision of values between samples with different PCR amplification efficiencies is possible because gene expression measurements are determined by the ratio of NT/CT for each gene and not by the absolute amount of NT PCR product. NT and CT for each gene are amplified with the same primers, share sequence homology, and amplify with equal efficiencies [1]. Therefore, differences in priming efficiency will not affect the measured relative level of expression between genes in different samples even after two rounds of amplification.

With multiplex StaRT-PCR, at least 96 gene expression measurements can be obtained from the same amount of cDNA typically used to obtain one gene

Table 2
Mean gene expression in universal human reference cDNA as measured by uniplex and multiplex StaRT-PCR

Gene	GenBank accession	Mean expression[a]		Gene	GenBank accession	Mean expression	
		Multiplex StaRT-PCR	Uniplex StaRT-PCR			Multiplex StaRT-PCR	Uniplex StaRT-PCR
HSD11B1	M76664/M68487	ND[b]	ND	GLUCT2	M57951	530	1000
ACHE	M55040	140	NA[c]	GSR	X15722	24,000	12,000
ALAS1	X56351	7300	17,000	GPX3	D00632	2500	NA
ARNT	M69238	8300	NA	GSTM1,2,4,5	J03817/M63509	16,000	19,000
BCL2	M14745	410	530	GSTM3	J05459	14,000	17,000
BCL2L1 (L)	Z23115/Z23116	3200	12,000	GSTP1	X06547	210,000	230,000
BCL2L1 (S)	Z23115/Z23116	6900	2300	GSTT1	X79389	NA	180
MYC	V00568	81,000	47,000	ERBB2	X03363	84,000	66,000
CALB2	X56667	NA	NA	HNF3A	U39840	360	730
CBR1	J04056/X51818	6700	34,000	HSPD1	XM_012182	130,000	NA
CAT	X04076	5400	7900	HSPA9B	L15189	17,000	13,000
CDC2	X05360	43,000	40,000	ICAM1	X06990	4300	6100
CDK7	X79193	6000	17,000	IVL	M13902	Low	Low
CDK8	X85753	1200	NA	JUNB	X51345	1300	11,000
GJA1	X52947	20,000	13,000	KRT5	M19723	ND	Low
JUN	J04111	60,000	210,000	IL16	M90391	Low	690
CLCN3	X78520	2600	4600	MAD	L06895	1200	1500
COL3A1	X14420	25	25	MAX	X60287	ND	ND
SOD1	X02317/K00065	220,000	130,000	MLH1	U07343	15,000	6000
CCNA2	X51688	4000	6700	MSH2	L47581	9200	9600
CCNG2	U47414	3600	3300	MSH6	U28946	180,000	57,000
CCNH	U11791	2100	3400	MSK2	AF074715	4300	2000
CYP1A1	K103191	540	240	MUC1	X52229	ND	ND
CYP1B1	U03688	1900	800	NADH	M28705	170,000	28,000
CYP2B6	M29874/J02864	ND	ND	POR	S90469	29,000	15,000
CYP2E1	J02843	48	18	NSE	M27610	76,000	62,000
CYP2F1	J02906	ND	ND	RBL2	X74594	1700	5000
DAO	X13227	96	59	CDKN2A	L27211	ND	Low
SULT2A1	U08024	2600	4700	CDKN2C	U17074	9300	17,000
DNASE 1	M55983	Low[d]	540	CDKN2D	U40343	1800	3500
TFDP2	U18422	13,000	20,000	CDKN1A	U03106	4000	9200
DPYS	D78011	57	22	CDKN1B	U10906	9000	8600
E2F1	M96577	2400	6,300	TP53	K03199	7200	3300
E2F2	L22846	1400	720	SAM68	M88108	190,000	130,000
E2F4	U15641	12,000	15,000	PCNA	J04719	120,000	54,000
E2F5	U15642	3200	2000	PMS2L3	D38437	1400	3900
ERCC1	M13194	61,000	25,000	SULT1A1	L19999	6100	4000
ERCC4	U64315	650	170	SULT1A3	U08032	28,000	22,000
EDN1	NM_001955	770	610	RAP1A	M22995	NA	38,000
FN1	X02761/K00799	360,000	140,000	RARA	X06614	4200	3200
GGH	U55206	18,000	14,000	RB1	M15400	3900	3600
FPGS	M98045	4200	2600	SPARC	J03040	320,000	110,000
FOSL1	X16707	40,000	92,000	SPR1B	M84757	18	62
GAPD	M33197	230,000	360,000	STX1A	L37792	1400	2400
GLCLC	M90656	380	1700	TGM2	M55153	NA	3200
GLI2	NM_005270	5000	1700	TNF	X02910/X02159	ND	ND
GLUCT1	M57899	Low	ND	TNFRF1A	M58286/M33480	2400	7600

[a] Molecules of mRNA/10^6 β-actin mRNA molecules.
[b] ND = None detected. No NT was detected when CT mix F was used.
[c] NA = Not assessed.
[d] Low = expression levels are less than 600 mRNA/10^6 β-actin mRNA.
Low expression is reported when no NT is seen when mix E was used and neither NT nor CT were measurable when mix F was used.

expression measurement using uniplex StaRT-PCR. Since the same amount of cDNA is used in round one of the multiplex process as used in uniplex StaRT-PCR, rare transcripts are not diluted out and can still be detected with statistical significance.

In uniplex StaRT-PCR, a sample of cDNA representing 100–1000 cells is typically used to measure one gene in one PCR. Using this amount, it is possible to detect transcripts that are expressed at 0.1–1 copy per cell (or 1–10 copies per 10 cells) with statistical signifi-

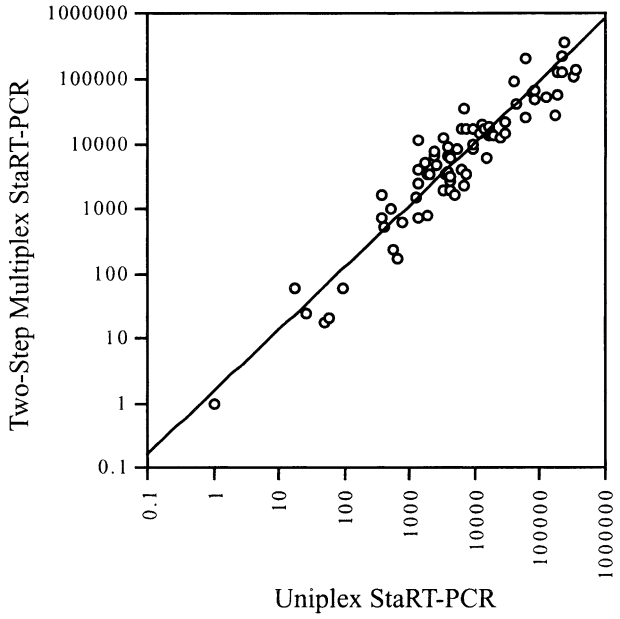

Fig. 3. Correlation of gene expression values obtained by either 96 gene Multiplex or Uniplex StaRT-PCR. Samples of cDNA derived from Stratagene Universal Human Reference RNA were combined with CT mix (mixes B, C, D, E, and F from GENE system 1 were used) and amplified either by uniplex StaRT-PCR or by 96 gene multiplex StaRT-PCR with primer pairs for all genes in GENE system 1. Mean values are presented in Table 2 for the 93 genes that could be evaluated (see text). Of these, 79 were measured by both uniplex and multiplex StaRT-PCRs and could be compared. Gene expression values are presented as molecules of mRNA per 10^6 β-actin mRNA molecules. Values obtained by uniplex StaRT-PCR are plotted along the *X* axis and values obtained by multiplex StaRT-PCR are plotted along the *Y* axis.

Table 3
Mean gene expression in primary bronchogenic carcinoma sample as measured by multiplex StaRT-PCR

Gene	GenBank accession #	Mean expression[a]	CV[b]
BCL2	M14745	ND[c]	–
TP53	K03199	5.6E + 02	0.37
MSH2	L47581	2.7E + 03	NA[d]
GSTM1,2,4,5	J03817/M63509	3.2E + 03	0.64
GSTM3	J05459	1.0E + 04	0.53
BCL2L1 (L)	Z23115/Z23116	1.8E + 04	0.16
MLH1	U07343	1.9E + 04	0.45
DNASE1	M55983	3.6E + 04	0.29
FPGS	M98045	4.9E + 04	0.30
ICAM1	X06990	5.7E + 04	0.32
E2F1	M96577	6.2E + 04	0.25
MSH6	U28946	6.4E + 04	0.24
BCL2L1 (S)	Z23115/Z23116	1.1E + 05	0.20
GJA1	X52947	1.1E + 05	0.15
CAT	X04076	1.8E + 05	0.22
ERCC1	M13194	5.1E + 05	0.26
SOD1	X02317/K00065	7.5E + 05	0.29
GSTP1	X06547	8.1E + 06	0.62

[a] Molecules of mRNA/10^6 β-actin mRNA molecules.
[b] Coefficient of variation.
[c] None detected. No NT was detected when CT mix F was used.
[d] Not applicable because only 2 expression values were obtained.

cance. This amount of cDNA typically is used in the first round of two round multiplex StaRT-PCR. Because this cDNA is co-amplified with CTs for each gene to be measured and since the relationship of endogenous cDNA to CT remains constant throughout PCR, PCR product from round one can be diluted and amplified again in a second round with primers specific to one gene without significantly changing the numerical values obtained relative to those obtained with uniplex StaRT-PCR. In this manner, sufficient PCR products can be generated to detect and measure gene expression for many genes without using additional cDNA (Tables 1–3). This will allow investigators to study more genes in and obtain more replicate data from small cDNA samples such as those that are available from biopsies, microdissected tissues or sorted cell populations. Here we have shown the feasibility of reproducibly measuring gene expression in an FNA biopsy of a lung tumor by multiplex StaRT-PCR (Table 3).

The method described here may enable marked reduction of round two reaction volumes, reagent consumption, and cost. This may be accomplished by using very small aliquots of round one multiplex StaRT-PCR products, instead of diluting as presented in Table 1. Efforts currently are underway to reduce PCR volumes from 10 to 10–100 μl. Combining this method with microfluidic CE should allow high-throughput measurement of gene expression in small tissue samples [16]. In contrast to previous attempts to measure expression of multiple genes using very small amounts of cDNA in multiplex [18], the method presented here is standardized and has the capability to detect rare transcripts without using expensive fluorescent probes. Since CTs are used in every reaction, false negatives do not occur. For these reasons, and because this method is highly compatible with automation, we believe multiplex StaRT-PCR will be a valuable tool in the clinical laboratory.

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