Journal of Molecular Diagnostics, Vol. 7, No. 4, October 2005

Scalable Transcriptional Analysis Routine—Multiplexed Quantitative Real-Time Polymerase Chain Reaction Platform for Gene Expression Analysis and Molecular Diagnostics

Elizabeth P. Garcia,*† Lori A. Dowding,* Lawrence W. Stanton,‡ and Vladimir I. Slepnev*†

From Primera BioSystems,† Providence, Rhode Island; Sention, Providence, Rhode Island; and Genome Institute of Singapore,‡ Singapore, Singapore*

We report the development of a new technology for simultaneous quantitative detection of multiple targets in a single sample. Scalable transcriptional analysis routine (STAR) represents a novel integration of reverse transcriptase-polymerase chain reaction and capillary electrophoresis that allows detection of dozens of gene transcripts in a multiplexed format using amplicon size as an identifier for each target. STAR demonstrated similar or better sensitivity and precision compared to two commonly used methods, SYBR Green-based and TaqMan probe-based real-time reverse transcriptase-polymerase chain reaction. STAR can be used as a flexible platform for building a variety of applications to monitor gene expression, from single gene assays to assays analyzing the expression level of multiple genes. Using severe acute respiratory syndrome (SARS) corona virus as a model system, STAR technology detected single copies of the viral genome in a two-gene multiplex. Blinded studies using RNA extracted from various tissues of a SARS-infected individual showed that STAR correctly identified all samples containing SARS virus and yielded negative results for non-SARS control samples. Using alternate priming strategies, STAR technology can be adapted to transcriptional profiling studies without requiring *a priori* sequence information. Thus, STAR technology offers a flexible platform for development of highly multiplexed assays in gene expression analysis and molecular diagnostics. *(J Mol Diagn 2005, 7:444–454)*

Nucleic acid testing of clinical samples and tissues is increasingly widespread and applied to various areas of medicine and diagnostics including pathogen identification,[1] blood bank testing,[2] cancer recurrence,[3] and prediction of clinical outcome.[4] The competitive landscape of available technologies is characterized by clear separation of very sensitive and quantitative methods measuring single bioanalytes [real-time polymerase chain reaction (PCR), transcription mediated amplification, ligase chain reaction, rolling circle amplification, and so forth] and methods capable of multiplexing thousands of genes (DNA microarrays, serial analysis of gene expression, differential display, and so forth) with less sensitivity and quantitative ability compared to single gene methods. The recent introduction of technologies capable of analyzing a number of analytes or biomarkers in a high-throughput multiplex configuration in clinical diagnostics[5,6] and gene expression analysis (HT Genomics, *www.htgenomics.com*) demonstrates a growing trend toward development of multiplex assays. The future of nucleic acid testing requires better multiplexing abilities that maintain or exceed the current levels of sensitivity.

Although real-time PCR is the most common method using optical detection, others such as transcription-mediated amplification, ligase chain reaction, and strand displacement amplification are also widely used and marketed for nucleic acid testing diagnostics.[7,8] Many of these techniques depend on the detection and quantification of fluorescent molecules whose signal increases in proportion to the amount of amplified nucleic acid generated. Yet multiplexing capabilities for these methods are limited due to the overlapping absorption and emission spectra of available fluorophores thus restricting the number of multiplexed targets to four or five.[9,10]

At the opposite extreme, several methods have been developed that have excellent multiplexing capabilities allowing the analysis of large amounts of genomic information. The most widely used method depends on DNA microarrays, a technique that detects expression of up to thousands of genes based on optical detection of hybridized fluorescently labeled DNA probes combined with spatial positioning.[11–13] Serial analysis of gene expression[14] and differential display[15] also allow the transcriptome of two samples to be compared with the added advantage that *a priori* sequence knowledge is not required. Despite this, each of these techniques has sev-

Supported by Sention, Inc.

Accepted for publication May 3, 2005.

Address reprint requests to Vladimir Slepnev, 4 Richmond Sq., Providence, RI 02906. E-mail: vslepnev@primerabio.com.

eral drawbacks. They lack sensitivity, show poor quantification capabilities, and require large amounts of biological material.[16] Finally, each is a long multistep process that requires a high level of technical expertise that can contribute to variability in data obtained.

We have developed STAR (scalable transcription analysis routine), a gene expression analysis platform that represents an innovative integration of real-time multiplex PCR and capillary electrophoresis (CE), allowing the simultaneous quantitative measurement of multiple targets in a single sample with high sensitivity. Specificity of PCR amplification is due to appropriate primer choice and reaction conditions. Because CE allows accurate size determination of fluorescently labeled nucleic acids from 50 to 1000 bases with single base precision, assays can be conducted simultaneously for dozens of targets whose identities are defined by the specific size of its corresponding amplicons, while maintaining quantification capabilities equal to or better than those observed with established real-time PCR methods. Although initial proof-of-concept experiments were performed manually, in the course of this work, we automated aliquot dispensing during PCR amplification, and are currently working on integration of a fully automated system that will integrate aliquot dispensing with CE separation. STAR is fast, cost-effective, and has a large dynamic range. Here we present STAR technology and its application to diagnostics and gene expression analysis.

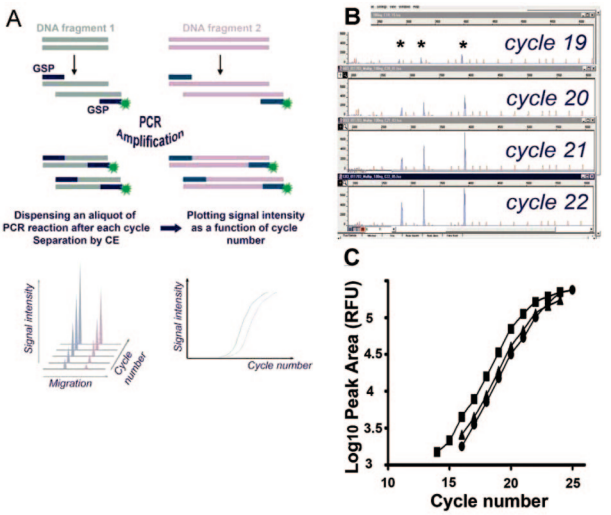

Figure 1. Description of STAR technology. **A:** Diagrammatic representation of STAR technology. See text for detailed explanation. Abbreviations: GSP, gene-specific primer; C_T, cycle threshold. To illustrate the process, three genes (arc, homer1a, and zif268) were amplified from 100 ng of rat brain total RNA in a multiplex format using 0.5 μmol/L of each gene-specific primer. Forward primers were fluorescently labeled. Aliquots collected between cycles 12 and 35 were separated by CE and analyzed by GeneScan version 3.7.1 generating electropherograms. **B:** Successive electropherograms from cycles 19 through 22 are shown. Peaks representing arc, homer1a, and zif268 are marked with an **asterisk**. Small repeating peaks represent DNA molecular size markers. **C:** Amplification curves for arc (**filled triangles**), homer1a (**filled circles**), and zif268 (**filled squares**) were reconstructed by plotting the area under each peak against cycle number.

Materials and Methods

Description of STAR Technology

In a typical STAR experiment (diagrammatically shown in Figure 1A), a PCR reaction is set up in a single tube containing the analyte, common PCR reagents (eg, DNA polymerase, dNTPs), and, for each target to be amplified, gene-specific primers where at least one of each pair is labeled with a fluorophore. PCR primers are designed for each target so that the amplicon length serves as a unique identifier for each particular target. Each amplicon must vary by at least 5 nucleotides in size. Aliquots of the multiplex PCR reaction are removed after successive PCR cycles and separated by CE. Amplification curves are reconstructed based on the area under each amplified target. As for conventional real-time PCR, cycle thresholds (C_T) can be determined from the graphs thereby allowing the determination of initial copy number for each amplified template. For applications that quantify RNA levels, STAR can be modified to a one-step reverse transcriptase (RT)-PCR reaction (see below).

One-Step STAR Protocol

RNA template and forward and reverse gene-specific primers were added to a mixture containing 1× Stratagene buffer (catalog no. 600532, modified to contain 0.1% Triton X-100, 1.5 mmol/L $MgCl_2$) and 0.3 U/μl of StrataScript RTase (Stratagene, La Jolla, CA) and reverse transcribed at 45°C for 50 minutes, followed by 2 minutes at 94°C. At least one of each primer pair was fluorescently labeled with FAM. The PCR protocol consisted of 31 to 44 cycles of 94°C for 30 seconds, 60°C for 30 seconds, and 72°C for 1 minute. While ramping up to the first 72°C extension, 1 U of Vent(Exo$^-$) DNA polymerase (New England Biolabs, Beverly, MA) was added. After a predetermined number of cycles, 3 μl aliquots were collected at the end of successive cycles and immediately added to 7 μl of formamide containing ROX-labeled DNA standards (BioVentures, Freesboro, TN) followed by heat denaturation and separation by CE using the 3100 Genetic Analyzer (Applied Biosystems, Foster City, CA). Samples were injected at 3 kV for 20 seconds and separated at 15 kV on POP4 polymer. Data were analyzed for peaks and relative areas as determined by Gene Scan version 3.7.1 software provided with the instrument. Data collected were analyzed to construct amplification curves using proprietary software, AnalySTAR, developed for Sention.

RNA Isolation from Rat Brain

Rats were anesthetized with isoflurane (Henry Schein, Melville, NY) and decapitated. Brains were quickly removed, flushed with oxygenated artificial cerebrospinal fluid, and kept cold during the dissection procedure. Extraction of total RNA was performed in a two-step protocol using Trizol (Invitrogen, Carlsbad, CA) and RNA easy kit (Qiagen, Valencia, CA) as per manufacturers' instructions.

Table 1. Nucleic Acid Sequence of Primers Used in This Study

Gene	Application	Primer sequence
*Homer1a**	TaqMan/STAR/SYBR	5′-CTGCTCCAAAGGAAAGCCTTGC-3 5′-AAACAACCTTCAATGCTGACGG-3′
	TaqMan probe	5′-[FAM]CGTCCTCTGTGGCACCTCTGTGGGC[TAMRA]−3′
*zif268**	TaqMan/STAR/SYBR	5′-GTTACCTACTGAGTAGGCGG-3′ 5′-TGAAGGATACACACCACATATC-3′
	TaqMan probe	5′-[FAM]CGCATTCAATGTGTTTATAAGCCA[TAMRA]-3′
*arc**	TaqMan/STAR/SYBR	5′-CCGACCTGTGCAACCCTTTC-3′ 5′-GCAGATTGGTAAGTGCCGAGC-3′
	TaqMan probe	5′-[FAM]TGCTTGGACACTTCGGTCAACAGATGCC[TAMRA]-3′
L-HA	Detection of artificial transcripts	5′-[FAM]CCATACGACGTCCCAGACTA-3′
pcDNA3L	Detection of artificial transcripts	5′-AGCTCTAGCATTTAGGTGACACTA-3′
inhibin	12plex	5′-CACACGGGGCTCGACAGGAAG-3′ 5′-[FAM]CCCCCAGATGACAGCACCAGAAG-3′
BMP14	12plex	5′-[FAM]ACTCCATCGGGCGCTTCTTTAG-3′ 5′-CAGGGAGCCGTAGTGGGTAGTTCT-3′
DGK	12plex	5′-[FAM]TCTGCCGAGCCCACATTGAG-3′ 5′-GGCGTCCAGGAAACACCACTTG-3′
actin	12plex	5′-[FAM]CACCCACACTGTGCCCAT-3′ 5′-TGGTGGTGAAGCTGTAGC-3′
LDH	12plex	5′-[FAM]AGCCCCGACTGCACCATCATC-3′ 5′-GTAACGAAACCGAGCAGAATCCAG-3′
PKCγ	12plex	5′-[FAM]TGCAGCCTCCTCCAGAAGTTTGA-3′ 5′-GTCCTGGGCTGGCACCGAAGAA-3′
EGR3	12plex	5′-CCGCAGCGACCACCTCACTAC-3′ 5′-[FAM]CACCCCCTTTCTCCGACTTCTTC-3′
18S rRNA	12plex	5′-[FAM]CGGCTACCACATCCAAGGAA-3′ 5′-GCTGGAATTACCGCGGCT-3′
PIPK	12plex	5′-CACCCCACCGTCCTTTGAG-3′ 5′-[FAM]ACCCCCACACCGCACACTG-3′
Nell2	12plex	5′-[FAM]GACAACACAACTGCGACAAAAATG-3′ 5′-GGCAGGTTAACACAGCGGGAGTAG-3′
arc	12plex	5′-CACCCTGCAGCCCAAGTTC-3′ 5′-[FAM]GCCCCAGCTCAATCAAGTCCTA-3′
NSE	12plex	5′-CGGCACGGGCAGGATGAG-3′ 5′-[FAM]TGGGGCAGCCGAGAAGGAC-3′
ST1	Alternative priming	5′-[FAM]CGCTCGTAGTCGAACGCCTAACCA-3′
ST2	Alternative priming	5′-[FAM, VIC, or NED]CGACGTATGCGTAACCCGTATCGT-3′
UT2	Alternative priming	5′-GCGGCGCCTATCTTACTAT
ST1-dT14-VN	Alternative priming	5′-CGCTCGTAGTCGAACGCCTAACCATTTTTTTTTTTTTTTVN-3′
ST2-dT14-VN	Alternative priming	5′-CGACGTATGCGTAACCCGTATCGTTTTTTTTTTTTTTTVN-3′
UT2-HA	Alternative priming	5′-GCGGCGCCTATCTTACTATCCAGACTA-3′
Rep1B	SARS multiplex	5′-AAGCCTCCCATTAGTTTTCCATTA-3′ 5′-[FAM]CACAACAGCATCACCATAGTCACC-3′
S	SARS multiplex	5′-ACGTCAGCTGCAGCCTATTTTGTT-3′ 5′-[FAM]TTGTCCTGGCGCTATTTGTCTTAC-3′

*For STAR experiments, the first oligo listed is FAM labeled; for SYBR and TaqMan, oligos are unlabeled.
FAM, 6-carboxyfluorescein; TAMRA, 6-carboxytetramethylrhodamine.

Construction of Artificial Constructs

HA-tagged amphiphysin gene constructs[17] were amplified using vector-specific 5′- and 3′-primers such that the T7 promoter was incorporated upstream of each construct. The 3′-primer contained a 15-nucleotide d(T) tail that allowed addition of a poly(A) during RNA preparation. PCR products were gel purified and transcribed using T7 RNA polymerase (Stratagene) generating artificial transcripts that contained common 5′ and 3′ nucleic acid sequences at each end and a poly(A) tail at the 3′end.

Primer Sequences

Primers for targets were designed using PrimerSelect (DNA Star, Madison, WI). Unlabeled primers were ordered from Qiagen, fluorescently labeled primers from Synthegen (Houston, TX) or Applied Biosystems, and TaqMan probes from Applied Biosystems. Primer sequences are shown in Table 1.

TaqMan Probe-Based and SYBR Green-Based Real-Time RT-PCR Protocols

Each sample was serially diluted 2-fold from the starting concentration in 0.1% bovine serum albumin and amplified in a one-step RT-PCR protocol using 0.5 μmol/L primers. For TaqMan probe-based real-time RT-PCR assays (TaqMan) using 0.066 μmol/L probe, cycling parameters were 1 cycle of 45°C for 45 minutes, 95°C for 10 seconds followed by 40 cycles of 95°C for 15 seconds, 57°C for 10 seconds, 57°C for 1 minute. SYBR Green-

based real-time RT-PCR (SYBR) cycling parameters were 1 cycle of 45°C for 30 minutes, 95°C for 7 minutes followed by 40 cycles of: 95°C for 30 seconds, 57°C for 30 seconds, 72°C for 1 minute. For both assays, the annealing temperature was reduced to 52°C for zif268 amplification. STAR cycling parameters were identical to that of SYBR except that RT was performed at 45°C for 50 minutes followed by 95°C for 2 minutes before PCR amplification. The annealing temperature for the multiplex STAR reaction was 55°C.

STAR Protocol Using Alternative Priming Strategies

For reverse transcription (RT), RNA template and sequence-tagged reverse primers (5 μmol/L) were added to 58% glycerol, heated at 70°C for 10 minutes, then put on ice for 2 minutes. Buffer (final concentrations: 50 mmol/L Tris-HCl, pH 8.3, 75 mmol/L KCl, 3 mmol/L $MgCl_2$, 0.01 mol/L dithiothreitol, 0.8 mmol/L dNTP, 0.2 mg/ml bovine serum albumin, 20% trehalose), 3.2 U/μl of Superscript II RNase H^- reverse transcriptase (SSRTII, Invitrogen) and 1 U/μl of RNAsin (Ambion, Austin, TX) were added and reverse transcribed at 45°C for 20 minutes, followed by denaturation at 75°C. A second round of RT at 48°C for 20 minutes was initiated with the addition of 50 U SSRTII followed by a third round of RT at 52°C for 20 minutes after a 2-minute 80°C denaturation step. Samples were alkaline treated with 0.04 mol/L NaOH (final concentration) for 15 minutes at 65°C, followed by addition of Tris, pH 7.5, to a final concentration of 70 mmol/L. Resultant cDNAs were purified using the QIAquick gel extraction kit (Qiagen) as per the manufacturer's instructions, except that 360 μl of QG buffer was added to each sample. For second strand synthesis, purified cDNA in 40 mmol/L Tris (pH 7.5), 20 mmol/L $MgCl_2$, 50 mmol/L NaCl, 0.2 mmol/L dNTPs was heat denatured at 95°C for 1 minute followed by addition of 1.6 μmol/L second strand primers and continued denaturation at 95°C for 4 minutes. The reaction was ramped to 37°C, 0.5 U/μl Sequenase DNA polymerase (USB, Cleveland, OH) was added and incubated for 1 hour. DNA was purified using the QIAquick gel extraction kit as above. Primers (0.5 μmol/L) were added and PCR amplification proceeded in 10 mmol/L KCl, 10 mmol/L $(NH_4)_2SO_4$, 20 mmol/L Tris-HCl (pH 8.8), 2 mmol/L $MgSO_4$, 0.1% Triton X-100, 0.2 mmol/L dNTPs, 20% Q solution (Stratagene), 2% dimethyl sulfoxide, 2 U Vent DNA polymerase overlaid with mineral oil. PCR protocol: 95°C for 5 minutes followed by a variable number of cycles of 95°C for 30 seconds, 56°C for 30 seconds, 72°C for 1 minute. Three-μl aliquots were collected at the end of each cycle for 24 successive cycles and processed as described.

SARS Samples

All RNA samples derived from SARS corona virus (SARS-CoV)-infected Vero cells,[18] blinded clinical samples A through H, and normal human tissues were obtained from the Genome Institute of Singapore. RNA extractions were performed using High Pure Viral RNA kit (Roche Diagnostics, Mannheim, Germany). The copy number of SARS-CoV RNA was quantified against reference standards using the LightCycler SARS-CoV quantification kit (Roche Diagnostics).

SARS Amplification by One-Step STAR

SARS-CoV RNA samples were diluted in *Escherichia coli* tRNA at 20 μg/ml. One-step amplification of SARS transcripts was performed as described above using appropriate fluorescently labeled forward and reverse primers for 1 cycle of 45°C for 50 minutes, 94°C for 2 minutes, followed by 44 cycles of 95°C for 30 seconds, 60°C for 30 seconds, 72°C for 1 minute.

Results

Proof of Principle: STAR Technology Can Be Used to Multiplex RT-PCR Transcripts from a Complex Background Generating Real-Time Amplification Curves

To illustrate the STAR process, a one-step multiplex amplification of three transcripts, *arc*,[19] *homer1a*,[20] and *zif268*[21] was performed from 100 ng of total rat brain RNA and analyzed. The upper primer of each primer pair was fluorescently labeled with FAM. Three-μl aliquots were collected after successive PCR cycles 12 through 35, separated by CE and analyzed. Peaks representing each of the amplified targets are depicted in sequential electropherograms derived from PCR cycles 19 through 22 (Figure 1B). Note the growing peaks with consecutive cycles and also the lack of nonspecific amplification. The area under the peaks corresponding to each target was plotted against cycle number to generate the amplification curves shown in Figure 1C. As shown, STAR technology clearly detected the presence of all three transcripts in a complex background generating typical RT-PCR amplification curves.

Comparison of STAR Technology to TaqMan Probed-Based and SYBR Green-Based Real-Time RT-PCR

STAR technology is a further extension of multiplex real-time PCR that allows a much greater degree of multiplexing compared to existing technologies. STAR benefits from the integration of PCR and CE in which specificity of PCR amplification is due to appropriate primer choice and reaction conditions, and CE allows resolution of DNA fragments that differ by a few bases. The multiplexing power of STAR is a result of using amplicon size as a unique identifier for particular genes or biomarkers instead of fluorescent color or melting temperature used in other methods. In STAR experiments, a multiplex PCR reaction is sampled after each PCR cycle and subsequently separated by CE. The area under peaks corre-

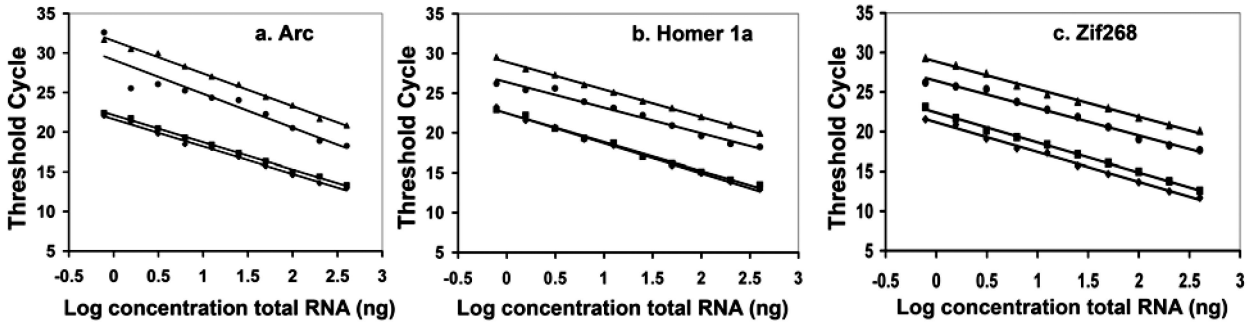

Figure 2. STAR technology is comparable to TaqMan probe-based and SYBR Green-based real-time RT-PCR. Detection of endogenous levels of *arc* (**a**), *homer1a* (**b**), and *zif268* (**c**) in rat brain total RNA were assessed by three real-time PCR methods: SYBR (**circles**), TaqMan (**triangles**), and STAR either as an individual reaction (**squares**) or as part of a multiplex reaction (**diamonds**). C_Ts were determined from PCR amplifications performed from twofold serially diluted total rat brain RNA (400 to 0.78 ng) and plotted. For SYBR and TaqMan, C_Ts were calculated using Bio-Rad ICycler software (Hercules, CA).

sponding to specific PCR products are quantified and plotted as a function of cycle number to generate amplification plots, which can be analyzed using current algorithms of real-time PCR analysis (Figure 1; see Materials and Methods for a complete description). Currently, TaqMan probe-based (TaqMan) and SYBR Green-based (SYBR) real-time RT-PCR are the methods of choice for detection and quantification of transcripts. We compared detection of three genes (*arc*, *homer1a*, and *zif268*) in a complex background using TaqMan, SYBR, and STAR, individually or as a three-gene multiplex. Twofold serially diluted samples of total brain RNA were used as sample templates. To allow direct comparison between protocols, the PCR primer sequences used for each assay were identical with the exceptions that TaqMan hydrolysis probe was added for TaqMan assays and the forward PCR primers were fluorescently labeled for STAR assays. Amplification efficiencies and amplification curves observed for STAR are similar to that of TaqMan and SYBR for all three genes examined, but with higher sensitivity (Figure 2, Table 2). Cycle thresholds are six and four cycles sooner for STAR protocols than for TaqMan or SYBR protocols, respectively, representing a 64-fold increase in STAR detection limits as compared to TaqMan or SYBR.

Amplification Efficiency Is Constant between Single Gene and Multigene Amplifications

One concern in multiplex analysis of transcripts by real-time RT-PCR is that the amplification efficiencies of given transcripts may be influenced by the presence of multiple transcripts undergoing amplification. Comparison of the amplification efficiencies observed for *arc*, *homer1a*, or *zif268* show that they are not significantly different whether performed in multiplex or as individual reactions (Table 2). We do, however, find that the cycle threshold is often lower in multiplexed samples than samples amplified individually. This is likely due to more efficient or promiscuous priming during reverse transcription resulting from the increased number of primers present in multiplex reactions that can anneal to RNA templates at low reaction temperatures (42°C; Figure 2).

Sensitivity and Reproducibility of STAR Assays

To address the absolute sensitivity of STAR assays, we performed experiments using artificial transcripts spiked into carrier tRNA (20 μg/ml; Sigma, St. Louis, MO). Three artificial transcripts [*VS31* (450 bases), *VS32* (377 bases), and *VS85* (544 bases)] were 10-fold serially diluted from 3,000,000 to 3 copies and PCR amplified in multiplex using a common pair of fluorescently labeled primers as each transcript contains common sequences at their 5′ and 3′ ends (see Materials and Methods). STAR detected 3 copies of each of the multiplexed transcripts and linearly detected samples from 3,000,000 to 30 copies for all transcripts with efficiencies ranging from 84.5 to 95.3%. Amplicons were not detected in negative controls.

To determine the precision of multiplex STAR assays within runs, we performed four identical reactions that contained 300 copies of each artificial transcript diluted in carrier RNA (Figure 3B). Cycle thresholds were 25.9 ± 0.48, 23.8 ± 0.44, and 26.8 ± 0.50 for *VS31*, *VS32*, and *VS85*, respectively, throughout the four runs demonstrating reliable reproducibility within STAR assays. Amplification curves are shown for *VS32* (Figure 3B). The mean amplification efficiency was 1.82 ± 0.06.

Table 2. Comparison of Three Real-Time Methods for Gene Expression Analysis

	homer1a			*zif268*			*arc*		
	Slope	Efficiency	r^2	Slope	Efficiency	r^2	Slope	Efficiency	r^2
TaqMan	−3.46 ± 0.05	94.33 ± 1.8	0.998	−3.45 ± 0.15	94.94 ± 5.4	0.996	−4.13 ± 0.10	74.73 ± 2.4	0.995
SYBR	−3.21 ± 0.16	104.8 ± 7.3	0.981	−3.49 ± 0.08	93.56 ± 3.0	0.986	−3.45 ± 0.37	94.88 ± 14	0.878
STAR single	−3.79 ± 0.09	83.41 ± 2.6	0.996	−3.65 ± 0.07	88.07 ± 2.4	0.997	−3.45 ± 0.09	95.10 ± 3.3	0.997
STAR multiplex	−3.61 ± 0.11	89.17 ± 3.8	0.992	−3.73 ± 0.07	85.34 ± 2.3	0.997	−3.43 ± 0.06	95.78 ± 2.4	0.995

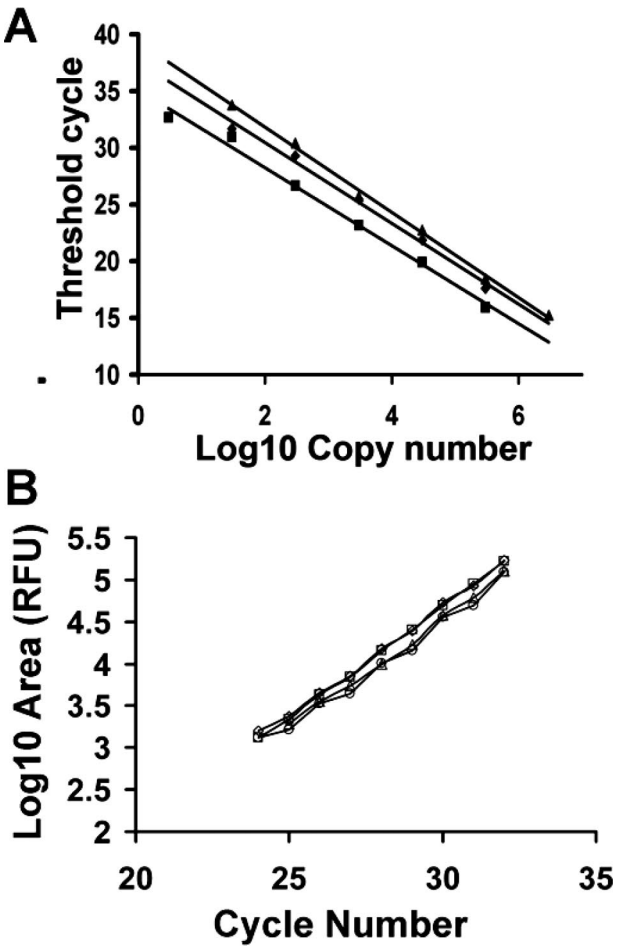

Figure 3. STAR is sensitive and reproducible. **A:** Artificial transcripts were 10-fold serially diluted from 3,000,000 to 3 copies in *E. coli* tRNA (20 μg/ml) and amplified as a three-gene multiplex STAR assay using (FAM)L-HA and (FAM)pcDNA3L primers. C_T versus copy number plots are shown for *VS31* (**diamonds**), *VS32* (**squares**), and *VS85* (**triangles**). Insufficient data points were obtained for *VS31* and *VS85* to allow C_T calculation at three copies. R^2 values were greater than 0.99. Amplification efficiency = $10^{(-1/\text{slope})} - 1$. **B:** To demonstrate reproducibility, four samples, each containing 300 copies of artificial transcripts *VS31*, *VS32*, and *VS85* in a background of *E. coli* tRNA (20 μg/ml), were multiplex amplified as above. Amplification curves for *VS32* are represented by **open circles**, **squares**, **triangles**, and **diamonds**.

Multiple Transcripts Covering a Wide Range of Expression Levels Can Be Amplified and Quantified Simultaneously in a Single Tube Using STAR Technology

To illustrate the multiplexing capacity of STAR, we performed simultaneous amplification of 12 transcripts from a sample of total rat brain RNA using a one-step STAR protocol (Figure 4). Targets were selected so that the predicted amplicon size was unique to each transcript. Cycle thresholds calculated for the various transcripts ranged from 1.3 for *18S* rRNA to 23.7 for *Nell-2* with mean amplification efficiencies of 73–92% demonstrating that even in the case in which the expression levels of 12 target genes varied over several orders of magnitude, transcripts were detected and amplified exponentially.

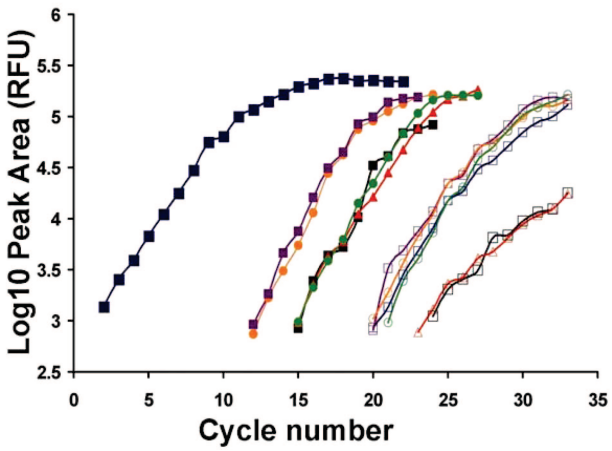

Figure 4. Multiplex amplification of 12 endogenous genes by STAR technology. Twelve endogenous genes were multiplex amplified using gene-specific primers from 400 ng of total rat brain RNA in a one-step STAR protocol. One of each primer pair was fluorescently labeled. Aliquots from cycles 2 to 33 were analyzed by CE to generate amplification curves shown. Abbreviations and symbols: *18S*, **dark blue closed squares**; *actin*, **purple closed squares**; *BMP*, **orange filled circles**; *inhibin*, **black closed squares**; *PKC*, **green closed circles**; *LDH*, **red closed triangles**, *DGK*, **purple open squares**; *EGR3*, **orange open circles**; *arc*, **blue open squares**; *PIPK*, **green open circles**; *Nell2*, **orange open triangles**; *NSE*, **black open squares**.

Gene Profiling Using Alternate Priming Strategies

Sample-to-Sample Comparisons

The STAR platform can be modified so that the gene expression patterns of two or more samples can be assessed in a single real-time PCR reaction if sequence tags[22] are incorporated into each set of primers. In the case of RNA analysis, each sample is separately reverse transcribed using composite primers consisting of a sequence tag (ST1 or ST2) fused to gene-specific sequences. The sequence tags act as identifiers for the sample source. Resultant cDNAs are pooled and PCR amplified using a simplified primer set: reverse primers ST1 and ST2, each labeled with a different fluorophore thereby allowing the sample source to be identified by specific color, and a gene-specific primer for each target effectively reducing the number of required PCR primers in half (*n* sequences require *n* + 2 PCR primers). This approach dramatically decreases the complexity of PCR amplification thus allowing multiplexing even greater numbers of genes/biomarkers. This approach was used to perform comparative real-time quantification of three transcripts in drug-treated versus saline-treated tissue samples normalized by comparing expression levels of *18S* rRNA. Results obtained using STAR technology confirmed the expression levels determined using microarray- and SYBR Green-based real-time RT-PCR (data not shown due to space limitation).

Transcriptional Profiling

With a few modifications the priming strategies described above can be adapted to transcriptional profiling of two samples even for unknown genes (Figure 5A).

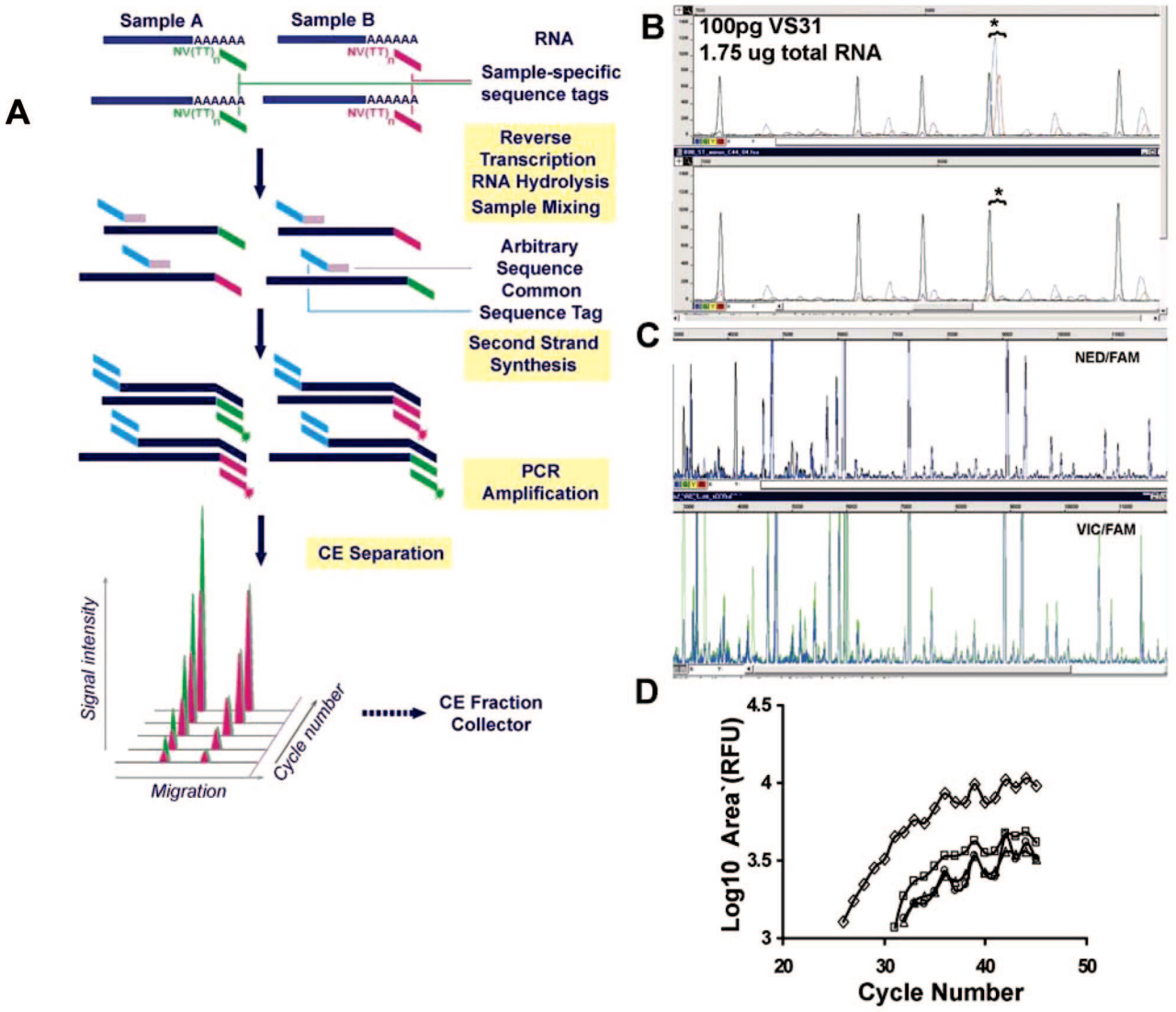

Figure 5. Alternative priming strategy using two sequence tags. **A:** Schematic representation depicting the incorporation of two sequence tags during RT-PCR. Incorporation of sample-specific sequence tags during RT is followed by incorporation of a second sequence tag during second strand synthesis. PCR then proceeds using three primers, each of the sample-specific reverse sequence tags fluorescently labeled, and the common forward sequence tag. Aliquots are collected, separated, and analyzed by CE followed by analysis of relevant fragments. **B:** Total rat brain RNA samples (1.75 μg) spiked with 100 pg of *VS31* (**top**) or unspiked (**bottom**) were separately reverse transcribed using sample-specific sequence tags (ST1-dT14-VN and ST2-dT14-VN) followed by RNA hydrolysis, purification, and sample mixing. Second strand synthesis was performed using UT2-HA followed by purification. Fragments were then amplified using FAM-ST1, ROX-ST2, and UT2. Aliquots were collected, separated by CE, and analyzed to generate the electropherograms shown. **C:** Total rat brain RNA (1.75 μg) was processed by STAR as described in **B** except that for PCR amplification NED-ST2 replaced ROX-ST2 (**top panel**) and VIC-ST2 replaced ROX-ST2 (**bottom**). **D:** Comparison of signal generated from 50 and 75 pg of input transcript. Two analytes were prepared in a background of 1.75 μg of total rat brain RNA. Analyte 1 was spiked with 50 pg of *VS31* and 50 pg of *VS85*. Analyte 2 was spiked with 50 pg of *VS31* and 75 pg of *VS85*. Each analyte was reverse transcribed separately using sample-specific sequence ST1dT14-VN or ST2dT14-VN generating cDNAs that were sequence tagged with either ST1 (analyte 1) or ST2 (analyte 2). Resulting cDNAs from both analytes were pooled and purified. Second strand was then synthesized using a forward sequence tag, UT2-HA. Again, excess primer was removed. Multiplex real-time PCR was performed using three primers, UT2, ROX-labeled ST1, and FAM-labeled ST2. Aliquots were taken from cycles 20 to 43 and analyzed by CE.

Samples are separately reverse transcribed using primers that contain a sample-specific sequence tag (ST1 or ST2) fused to poly(T)$_n$-VN that anchors the primer to the 5′-end of the poly(A) tail. Samples are then mixed followed by second strand synthesis reaction using forward primers that combine a second sequence tag (UT2) fused to a short arbitrary nucleotide sequence (hexamer or octamer) that can prime at many loci. Based on random occurrence, hexamers should occur every 4096 bases and octamers every 65,536 bases. However, the frequency of hexamers in human expressed genome is not random and shows considerable bias (V. Cheung, personal communication). By selecting arbitrary sequences with care, a series of second strand primers can be developed that result in amplification of hundreds to thousands of cDNAs per reaction based on the current expressed sequence tag database. We estimate that 95% of the expressed genome can be covered with 20 second strand primers coupled to hexamers. Samples are then PCR amplified using reverse primers FAM-

labeled ST1 and ROX-labeled ST2, and forward primer UT2.

As proof of principle, we analyzed the amplification profiles of two identical rat brain RNA samples that had been spiked with *VS31*, an artificial transcript of defined size. Analysis of electropherograms demonstrates that, as expected, a similar series of transcripts are amplified including the spiked transcript (Figure 5B; top, asterisk). Unexpectedly, we find that ROX labeling introduced a 2-base shift in each of the analyzed fragments. To demonstrate that residual peaks were not due to nonspecific priming of spiked *VS31*, the experiment was repeated without spiking (Figure 5B, bottom). As shown, the *VS31* fragment is absent while background peaks amplified from the total RNA background are still present.

Further experiments explored the feasibility of using other fluorophore combinations. Two identical samples of total RNA were subjected to STAR analysis as described above, except that for PCR amplification ROX-labeled ST2 was replaced with NED-labeled ST2 (Figure 5C, top) or VIC-labeled ST2 (Figure 5C, bottom). We found that both fluorophores NED and VIC could be used in combination with FAM and, unlike ROX, do not produce a shift in peak size. Using these labels, we observed completely overlapping peaks (Figure 5C).

To determine the differentiating ability of STAR using sequence tags, experiments were performed to analyze varying quantities of spiked artificial transcripts in the background of total rat brain RNA. Fifty pg of *VS31* was spiked into two rat RNA samples, whereas amount of *VS85* differed 1.5-fold in these samples. Analysis of the amplification plot (Figure 5D) demonstrated that, as expected, equal amounts of target RNA were amplified with the same kinetics. At the same time, a 1.5-fold difference in the level of RNA transcript could be easily detected by amplification kinetics or threshold cycle measurement.

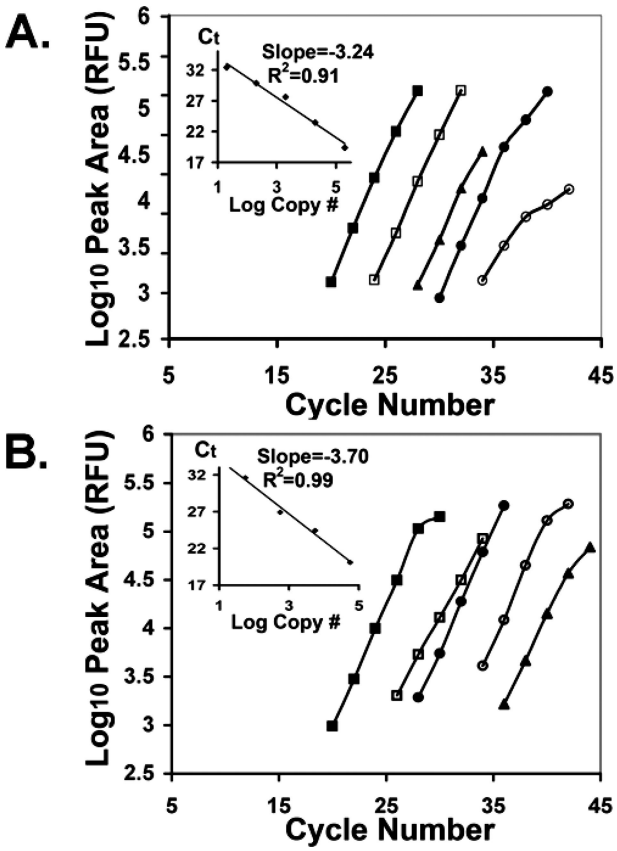

Figure 6. Detection of purified SARS-CoV RNA spiked into human biological samples. Samples were 10-fold serially diluted from their original concentration to <10 copies per reaction and amplified by one-step multiplex RT-PCR STAR protocol for the *S* and *Rep1B* genes. Aliquots from cycles 20 to 43 were analyzed by CE to generate amplification curves. Data for the *S* gene are shown for stool-derived samples (**A**) [200,000 (**filled squares**), 20,000 (**open squares**), 2000 (**filled triangles**), 200 (**filled circles**), and 20 (**open circles**) copies] or sputum-derived samples (**B**) [56,000 (**filled squares**), 5600 (**open squares**), 560 (**filled circles**), 56 (**open circles**), and 5.6 (**filled triangles**) copies]. **Insets**: C_T versus copy number graphs derived from data in **A** and **B**.

Diagnostic Applications of STAR Technology

For diagnostic purposes, a one-step reaction is the preferred method because it minimizes manipulations that can lead to errors or contamination of samples. We selected five primer pairs for SARS-CoV, each specific for one of the major genes.[23,24] These primer pairs were evaluated in a multiplex STAR assay using a plasmid library spanning the SARS-CoV genome. Primers detecting the Rep1B and S genes that showed the best amplification efficiencies were selected for a one-step multiplex RT-PCR protocol. During this process we found that replacing *Taq* polymerase with Vent(Exo$^-$) polymerase significantly improved the detectability of PCR products (data not shown). This may be due to better thermostability of Vent polymerase and its lack of 3′ exonuclease activity that may result in slow degradation of 5′-labeled primers and PCR products.[25] Because Vent polymerase had not been previously used in one-step RT-PCR reactions, we optimized the assay conditions to be compatible with Vent.

Detection of SARS-CoV RNA Using STAR Technology

Using our optimized assay, both *S* and *Rep1B* gene targets were detected from 500,000 to 5 copies using SARS-CoV RNA isolated from infected cultured Vero cells (data not shown). The assay was further validated using mock clinical samples created by serial dilution of purified SARS-CoV RNA into purified RNA samples obtained from uninfected donors (Figure 6). The amount of SARS-CoV RNA spiked into normal human RNA isolated from stool or sputum was in the range of 5.6 to 56,000 or 2 to 200,000 copies per reaction, respectively. Unspiked RNAs served as negative controls. One-step multiplex RT-PCR was performed on each of the samples and analyzed by the STAR protocol. Amplification curves and cycle threshold plots are shown for one of the multiplexed genes, *S*, detected from stool and sputum RNA. Similar results were obtained for *Rep1B* (data not shown). In stool samples, 5.6 copies were easily detected (Figure 6A) whereas 20 copies were detected from sputum (Figure 6B). Although we did not detect SARS-CoV RNA at two

copies in sputum samples, rather than lack of sensitivity, this may reflect the statistical absence of viral RNA in the reaction. C_T plots show good amplification efficiencies for the S gene (100%) whether performed in a background of human sputum or stool RNA (Figure 6, insets). Quantitative differences in C_T values for sputum and stool reflect differences in RT efficiencies between RNA samples derived from different tissues.

SARS-CoV Detection from Blinded Clinical Samples

To further validate STAR, true clinical samples from several tissue sources showing variable titers of SARS virus were supplied as blinded samples and assessed for the presence of SARS-CoV. Results of the STAR assay were then compared with those obtained using the Roche LightCycler quantification kit. Both SARS-CoV target sequences, *S* and *Rep1B*, were correctly detected in four tissue samples (intestine, lymph node, spleen, and throat swab) obtained from an individual who had contracted SARS and subsequently died from the disease. Quantitative RT-PCR indicated that these RNA samples contained 39,900 to 162 viral RNA copies/μl. Cycle thresholds calculated for both *Rep1B* and *S* showed good reproducibility for each of the tissues (data not shown). Four blinded control samples were negative as assessed by STAR and the Roche LightCycler quantification kit.

Discussion

We have developed a novel PCR-based, highly accurate, and reliable method for multiplexed analysis of nucleic acids suitable for gene expression studies and clinical diagnostics. This method combines the quantitative capacity of real-time PCR with the vast separating power of CE allowing simultaneous measurement of dozens of DNA or RNA targets in a single reaction. Integration of PCR and CE for multiplexed assays was originally suggested as end-point PCR amplification followed by analysis and quantification of the amplified targets by CE in which each target DNA was identified based on amplicon length.[26] This approach was successfully applied to gene expression analysis[22,27] and diagnostic applications[28–31] using end-point PCR detection. However, real-time PCR is preferred to endpoint PCR due to better precision and broader dynamic range of quantitative measurements.[32] Li and colleagues[33] furthered the technology demonstrating that parallel single gene PCR reactions terminated after successive cycles and analyzed by CE results in amplification curves for real-time analysis of a single target. STAR technology extends this concept to enable real-time PCR algorithms for quantification of multiple targets within a single reaction. Rather than setting up separate PCR reactions for each aliquot, STAR monitors PCR amplification by withdrawing an aliquot from the same reaction tube after each successive cycle. This arrangement decreases the total volume required for PCR, avoids variability caused by nonuniform thermocycling across PCR plates, and permits simpler design of an analytical instrument (for example by using direct electrokinetic transfer from PCR tube to the capillary).

A direct comparison of STAR to the two current standards for gene expression analysis, TaqMan and SYBR showed that STAR's ability to quantify gene transcripts was equal to these methods. We find that PCR efficiencies observed for each of the methods are within the range of experimental error (Table 2) and are likely to reflect the different fluorescent labeling methods used. We have previously found that amplification efficiencies can be differentially affected by covalent modifications of primers (data not shown). TaqMan assays can also show variable PCR efficiencies based on nucleotide composition, hybridization kinetics, and probe positioning.[34] This result is in good agreement with other multiplex real-time PCR methods in which efficiency of multiplex PCR could be optimized to the level of single gene amplification.[35] We also observed consistently lower threshold cycles for STAR assays than for TaqMan and SYBR assays. This observation can be partly explained by differences in the detection methods used (laser-induced fluorescence reading using ABI 3100 Prism instrument, versus charge-coupled device camera readings on the Bio-Rad ICycler real-time PCR system). However, there is a fundamental difference in the sensitivity of STAR and other optical real-time PCR methods due to reaction mechanisms. STAR directly measures fluorescence of the PCR product, whereas TaqMan and SYBR assess a secondary reaction that relies on an interaction between the PCR product and a signal-generating probe or dye, respectively. It is therefore not unexpected that methods based on the detection of primary PCR products are more sensitive than methods based on secondary signal-generating probe or dye intercalation.

STAR has several other advantages over current real-time PCR methods, especially for multiplex applications. First, STAR tolerates a much broader range of amplicon size (50 to 1000 bases with current ABI CE systems) than current real-time PCR methods (generally limited to 70 to 200 bases) enabling efficient choice of primer pairs that are compatible based on shared physical properties and composition. For targets that show high sequence variability such as HIV-1,[36] a broader scope of options for primer design is available. Second, because detection does not require probe hydrolysis, all restrictions related to the probe design (probe length and composition, distance to PCR primers) do not influence primer selection. Third, STAR technology has the potential to analyze hundreds of targets per reaction due to discrimination of DNA fragments both by size and fluorophore color. Current real-time PCR methods are limited to four targets per reaction. Finally, as specific PCR products are well separated from potential nonspecific PCR products (for example, primer-dimers), primer design criteria for STAR protocols can be relaxed.

The potential of STAR for larger scale multiplexing is illustrated by real-time amplification of 12 genes in a single RT-PCR reaction. We have also developed an in-house single nucleotide polymorphism assay that multiplexes 20 alleles within a single reaction (data not shown). To our knowledge, these are the first examples

of real-time PCR of more than 10 genes simultaneously. Importantly, development of this assay required no primer optimization because the first set selected by commonly used primer design software was successfully implemented. Moreover, multiplexing with STAR is only limited by the separation power of CE, which can separate hundreds of DNA fragments differing by a single nucleotide in size. However, expanding STAR multiplex assays to include dozens to hundreds of targets using gene-specific primers can result in severe background due to primer interactions and nonspecific amplification. The alternate priming strategies outlined that result in incorporation of sequence tags dramatically simplifies the PCR reaction by reducing the number of primers required, thereby decreasing background amplification.

Unlike current multiplexed RT-PCR methods, STAR can also detect and quantify changes in gene expression patterns even if the identity of the gene is unknown. Multiplexed RT-PCR methods using TaqMan or Molecular Beacons require *a priori* sequence knowledge of each target assayed. Using sequence tags and random hexamers as outlined in Figure 5, we have observed simultaneous real-time amplification of ~150 different DNA fragments using STAR for direct transcriptional profiling (Figure 5, B and C) and have found that the patterns obtained from total rat brain RNA are reproducible (data not shown). Zhong and Yeung[37] have similarly found that tissue-specific patterns of cDNAs are generated when RNA is reverse transcribed using random hexamers. We anticipate that a genetic fingerprint can be defined for various tissues for each second strand primer used. Each fragment can be predicted from available genomic sequence data or identified by sequencing to define the expressed transcriptome for each of the tissues. Because the real-time quantitative algorithm used in STAR provides better quantification of small differences as well as a much broader dynamic range allowing broadly expressed transcripts to be analyzed, this database could serve as a diagnostic tool because the presence/absence and expression level of each fragment would be known. Novel amplicons may represent overexpressed genes, products of gene fusion, or *trans*-splicing, alternatively spliced genes, or genes derived from pathogens. The use of multiple fluorophores also allows comparison of at least two samples during transcriptional profiling suggesting that STAR would be comparable to microarrays with the added advantage of identifying new genetic transcripts.

Besides applications for gene expression analyses, multiplexed assays using STAR protocols offer considerable advantages for molecular diagnostics. In addition to the obvious consideration of cost, multiplexed assays require less sample material to be withdrawn from patients, and much simpler logistics regarding laboratory tests. As a first step in the development of multiplexed diagnostic assays based on STAR technology we designed an assay for quantitative measurement of SARS-CoV. This assay was developed as a one-step RT-PCR to detect and quantify SARS-CoV RNA throughout a broad concentration, ranging from several viral copies up to several hundred thousand viral copies per reaction. In a blinded study, our assay correctly identified both SARS-CoV target sequences from tissue samples obtained from a SARS-infected individual with titers ranging from 39,900 to 162 viral copies per reaction without showing any false-positives. The STAR assay for SARS-CoV detection used simultaneous amplification of several target sequences from the SARS-CoV genome to confirm specificity of the assay especially in the context of complex clinical samples. This assay serves as a prototype for diagnostic tests in which a single clinical sample can be assessed for the presence of several pathogens. Alternatively, targeting several regions of the pathogen genome may be necessary for detection of rapidly mutating organisms because use of a single pair of specific primers may lead to false-negatives due to mutation of that site. This could be particularly important in the case when mutations are associated with virulence or drug resistance.[38]

STAR technology could be used for many immediate applications, most of which require multiplexing in the range of several dozen genes rather than hundreds. Such applications may include quantitative analysis of gene sets comprising a molecular signature for a biological process, effects of drug treatment on gene expression, and transcriptional signatures of disease conditions (identified in transcriptional profiling studies using DNA microarrays). An added benefit of the STAR approach is its scalability and flexibility regarding multigene assays. Any STAR assay can be easily updated by addition or subtraction of particular primer pair(s) as long as there is no overlap in amplicon size. Finally, the STAR protocol is adaptable to high-throughput automation that in its simplest form would include a dispensing thermocycler coupled to CE.

These considerations will multiply for analyses required for biodefense applications such as: medical surveillance; automated air, food, and water monitoring; and agricultural surveillance. All of these applications should have the capacity to identify and to measure multiple pathogens in a single sample with high sensitivity in a cost-effective manner. The potential of STAR could be realized through the direct integration of PCR with ultrafast CE separation. As a first step in STAR automation, we assembled a breadboard of the dispensing thermocycler to facilitate accurate sampling of PCR reaction. This system is based on the integration of a robotic fluid dispenser and a remotely operated thermocycler that will be described elsewhere. A specialized instrument that integrates PCR thermocycling, sample dispensing, and capillary-based separation is currently under development. Aside from instrument design, STAR development would also require a comprehensive bioinformatics package spanning multiplex primer design to management of gene expression data generated by STAR. As a first step, we developed a prototype software analysis program that facilitates the conversion of electropherogram data into real-time amplification plots thereby reducing the time required to analyze STAR-generated data from days to minutes. The features and benefits described above make the STAR approach a natural candidate for development of a broad array of multiplex assays.

Acknowledgments

We thank Drs. Randall Carpenter, Kazumi Shiosaki, Leon Cooper, Mark Bear, Mel Epstein, and Stan Rose for supporting this project at Sention; Dr. Edward Yeung for helpful discussions regarding capillary electrophoresis; Tim Clark, Marco Ocana, and Georgios Asteris for development of software tools for data analysis; Dr. Vivian Cheung for statistical analysis of nucleotide sequences; and Lisa Ng and Se Thoe Su Yun for SARS-CoV sample preparation.

References

1. Woods GL: Molecular techniques in mycobacterial detection. Arch Pathol Lab Med 2001, 125:122–126
2. Roth WK, Weber M, Petersen D, Drosten C, Buhr S, Sireis W, Weichert W, Hedges D, Seifried E: NAT for HBV and anti-HBc testing increase blood safety. Transfusion 2002, 42:869–875
3. Paik S, Shak S, Tang G, Kim C, Baker J, Cronin M, Baehner FL, Walker MG, Watson D, Park T, Hiller W, Fisher ER, Wickerham DL, Bryant J, Wolmark N: A multigene assay to predict recurrence of tamoxifen-treated, node-negative breast cancer. N Engl J Med 2004, 351:2817–2826
4. Nashed AL, Rao KW, Gulley ML: Clinical applications of BCR-ABL molecular testing in acute leukemia. J Mol Diagn 2003, 5:63–72
5. Vargo J, Smith K, Knott C, Wang S, Fang C, McDonough S, Giachetti C, Caglioti S, Gammon R, Gilbert D, Jackson JB, Richards W, Stramer S, Mimms L: Clinical specificity and sensitivity of a blood screening assay for detection of HIV-1 and HCV RNA. Transfusion 2002, 42:876–885
6. Pehler-Harrington K, Khanna M, Waters CR, Henrickson KJ: Rapid detection and identification of human adenovirus species by Adenoplex, a multiplex PCR-enzyme hybridization assay. J Clin Microbiol 2004, 42:4072–4076
7. Iqbal SS, Mayo MW, Bruno JG, Bronk BV, Batt CA, Chambers JP: A review of molecular recognition technologies for detection of biological threat agents. Biosens Bioelectron 2000, 15:549–578
8. Vernet G: Molecular diagnostics in virology. J Clin Virol 2004, 31:239–247
9. Kricka L: Stains, labels and detection strategies for nucleic acids assays. Ann Clin Biochem 2002, 39:114–129
10. Lee LG, Connell CR, Bloch W: Allelic discrimination by nick-translation PCR with fluorogenic probes. Nucleic Acids Res 1993, 21:3761–3766
11. DeRisi JL, Iyer VR, Brown PO: Exploring the metabolic and genetic control of gene expression on a genomic scale. Science 1997, 278:680–686
12. Schena M, Heller RA, Theriault TP, Konrad K, Lachenmeier E, Davis RW: Microarrays: biotechnology's discover platform for functional genomics. Trends Biotechnol 1998, 16:217–218
13. Bodrossy L, Sessitsch A: Oligonucleotide microarrays in microbial diagnostics. Curr Opin Microbiol 2004, 7:245–254
14. Tuteja R, Tuteja N: Serial analysis of gene expression (SAGE): unraveling the bioinformatics tools. Bioessays 2004, 26:916–922
15. Bartlett JM: Differential display: a technical overview. Methods Mol Biol 2003, 226:217–224
16. Luo Z, Geschwind DH: Microarray applications in neuroscience. Neurobiol Dis 2001, 8:183–193
17. Slepnev VI, Ochoa GC, Butler MH, DeCamilli P: Tandem arrangement of the clathrin and AP-2 binding domains in amphiphysin 1 and disruption of clathrin coat function by amphiphysin fragments comprising these sites. J Biol Chem 2000, 275:17583–17589
18. Tan ELC, Ooi EE, Lin C-Y, Tan HC, Ling AE, Lim B, Stanton LW: Inhibition of SARS coronavirus infection in vitro with clinically approved antiviral drugs. Emerg Infect Dis 2004, 10:581–586
19. Link W, Konietzko U, Kauselmann G, Krug M, Schwanke B, Frey U, Kuhl D: Somatodendritic expression of an immediate early gene is regulated by synaptic activity. Proc Natl Acad Sci USA 1995, 92:5734–5738
20. Ango F, Prezeau L, Muller T, Tu JC, Xiao B, Worley PF, Pin JP, Bockaert J, Fagni L: Agonist-independent activation of metabotropic glutamate receptors by the intracellular protein Homer. Nature 2001, 411:962–965
21. Milbrandt J: A nerve growth factor-induced gene encodes a possible transcriptional regulatory factor. Science 1987, 238:797–799
22. Johnson PH, Walker RP, Jones SW, Stephens K, Meurer J, Zajchowski DA, Luke MM, Eeckman F, Tan Y, Wong L, Parry G, Morgan TJ, McCarrick M, Monforte J: Multiplex gene expression analysis for high-throughput drug discovery: screening and analysis of compounds affecting genes overexpressed in cancer cells. Mol Cancer Ther 2002, 1:1293–1304
23. Marra MA, Jones SJ, Astell CR, Holt RA, Brooks-Wilson A, Butterfield YSN, Khattra J, Asano JK, Barber SA, Chan SY, Cloutier A, Coughlin SM, Freeman D, Girn N, Griffith OL, Leach SR, Mayo M, McDonald H, Montgomery SB, Pandoh PK, Petrescu AS, Robertson AG, Schein JE, Siddiqui A, Smailus DE, Stott JM, Yang GS, Plummer F, Andonov A, Artsob H, Bastein N, Bernard K, Booth TF, Bowness D, Czub M, Drebot M, Fernando L, Flick R, Garbutt M, Gray M, Grolla A, Jones S, Feldmann H, Meyers A, Kabani A, Li Y, Normand S, Stroher U, Tipples GA, Tyler S, Vogrig R, Ward D, Watson B, Brunham RC, Krajden M, Petric M, Skowronski DM, Upton C, Roper RL: The genome sequence of the SARS-associated coronavirus. Science 2003, 300:1399–1404
24. Ruan YJ, Wei CL, Ee LA, Vega VB, Thoreau H, Yun STS, Chia J-M, Ng Patrick, Chiu KP, Lim L, Tao Z, Peng CK, Ean LOL, Lee NM, Sin LY, Ng LFP, Chee RE, Stanton LW, Long PM, Liu ET: Comparative full-length genome sequence analysis of 14 SARS coronavirus isolates and common mutations associated with putative origins of infection. Lancet 2003, 361:1779–1785
25. Mattila P, Korpela J, Tenkanen T, Pitkanen K: Fidelity of DNA synthesis by the Thermococcus litoralis DNA polymerase—an extremely heat stable enzyme with proofreading activity. Nucleic Acids Res 1991, 19:4967–4973
26. Martin F, Vairelles D, Henrion B: Automated ribosomal DNA fingerprinting by capillary electrophoresis of PCR products. Anal Biochem 1993, 214:182–189
27. Odin E, Wettergren Y, Larsson L, Larsson PA, Gustavsson B: Rapid method for relative gene expression determination in human tissues using automated capillary gel electrophoresis and multicolor detection. J Chromatogr B Biomed Sci Appl 1999, 734:47–53
28. Lim EL, Tomita AV, Thilly WG, Polz MF: Combination of competitive quantitative PCR and constant-denaturation capillary electrophoresis for high-resolution detection and enumeration of microbial cells. Appl Environ Microbiol 2001, 67:3897–3903
29. Porsche MO, Braden D, Stephens K: Diagnosis of five spinocerebellar ataxia disorders by multiplex amplification and capillary electrophoresis. J Mol Diagn 2002, 4:108–113
30. Lawrence ER, Griffiths DB, Martin SA, George RC, Hall LM: Evaluation of semiautomated multiplex PCR assay for determination of Streptococcus pneumoniae serotypes and serogroups. J Clin Microbiol 2003, 4:601–607
31. Erdman DD, Weinberg GA, Edwards KM, Walker FJ, Anderson BC, Winter J, Gonzalez M, Anderson LJ: GeneScan reverse transcription-PCR assay for detection of six common respiratory viruses in young children hospitalized with acute respiratory illness. J Clin Microbiol 2003, 41:4298–4303
32. Wilhelm J, Pingoud A: Real-time polymerase chain reaction. ChemBioChem 2003, 4:1120–1128
33. Li N, Tan WG, Tsang RY, Tyrrell DL, Dovichi NJ: Quantitative polymerase chain reaction using capillary electrophoresis with laser-induced fluorescence detection: analysis of duck hepatitis B. Anal Bioanal Chem 2002, 374:269–273
34. Lunge VR, Miller BJ, Livak KJ, Batt CA: Factors affecting the performance of 5′ nuclease PCR assays for Listeria monocytogenes detection. J Microbiol Methods 2002, 51:361–368
35. Vet JA, Majithia AR, Marras SA, Tyagi S, Dube S, Poiesz BJ, Kramer FR: Multiplex detection of four pathogenic retroviruses using molecular beacons. Proc Natl Acad Sci USA 1999, 96:6394–6399
36. Malim MH, Emerman M: HIV-1 sequence variation: drift, shift, and attenuation. Cell 2001, 104:469–472
37. Zhong W, Yeung ES: High-throughput analysis of total RNA expression profiles by capillary gel electrophoresis. Anal Chem 2003, 75:4415–4422
38. Gardner SN, Kuczmarshki TA, Vitalis EA, Slezak TR: Limitations of TaqMan PCR for detecting divergent viral pathogens illustrated by hepatitis A, B, C, and E viruses and human immunodeficiency virus. J Clin Microbiol 2003, 41:2417–2427