

# StaRT-PCR™

## Quality-Controlled, Multi-Gene Expression Measurement for Development of Drugs, Biomarkers and Molecular Diagnostics

*StaRT-PCR™* (Standardized Reverse Transcription Polymerase Chain Reaction) is a patented, standardized, quantitative, next-generation method for measuring multi-gene transcript abundance in biological samples (1, 2, 3). *StaRT-PCR™* measures transcript abundance by utilizing Standardized Mixtures of Internal Standards™ (SMIS™) in every expression measurement. The SMIS™ contain fixed molar ratios of gene-specific internal standards for each gene measured, thus allowing for many advantages and benefits over all other gene expression methods (Table 1).

Transcript abundance measurement or transcript profiling allows scientists to identify a gene or a group of genes (biomarkers) relevant to a cellular pathway or specific disease and facilitates the drug discovery, validation and developmental processes. We at Gene Express, Inc., are gene expression specialists serving researchers worldwide with *StaRT-PCR™* that measures transcript abundance with SMIS™ that contain internal standards for each gene in every expression measurement. We consistently deliver higher quality and more reproducible data than any other method through our Standardized

Expression Measurement (SEM) Center™. *StaRT-PCR™* is the ideal method to measure gene expression for many reasons.

### Integrated Quality Control through Internal Standards with SMIS™

Universal internal standards specific for each gene are included in every assay in the form of a SMIS™. This provides for integrated quality control and quality assurance in the *StaRT-PCR™* method, since the standard is measured in the same environment as the test analyte (the transcript to be measured). This unique benefit of *StaRT-PCR™* controls for (A) reaction-to-reaction variability, (B) common sources of variation in PCR amplification and (C) inhibition of the PCR reaction. In addition, the SMIS™ allow for inter-sample comparisons and intra-sample comparisons that other methods cannot provide.

*StaRT-PCR™* measurements are truly quantitative. Results for all genes in a sample are standardized to their respective, precise internal standards in the SMIS™. Thus, the resultant data are independent values based on units of expression (molecules of target gene to molecules of normalizer gene) and not dependent on a reference sample required by relative or semi-quantitative gene expression measurements.

### Standardized Molecular Results

All *StaRT-PCR™* data are presented as number of molecules of gene transcript per number of molecules of a reference or normalizer gene. The employment of SMIS™ produces standardized data. The number of molecules for each transcript is determined based on the measurement of an internal standard of precisely known concentration

**Table 1: Clear Benefits of *StaRT-PCR™* Over Other Gene Expression Measurement Technologies**

#### Integrated Quality Control with SMIS™

- Controls for Sources of Variation in PCR
- Internal Standards as SMIS™ Provide a Unit of Measurement for True Quantitative Comparison

#### Standardized Molecular Results

- Inter- and Intra-Institutional Comparison of Data
- Multi-Gene Analysis
- Universal Gene Expression Database

#### Ruggedness of Methodology

- No False Negatives of PCR Reactions
- Statistically Insignificant False Positives through Stringent SOPs
- Provides FDA Good Laboratory Practices (GLP) Compliant Results

#### Accurate Quantitative Results

- Measures Number of Gene Transcript Molecules
- Gene Specificity through Gene-Specific Primers and Verified Amplimer Sizes

#### Excellent Reproducibility

- CVs average 10%

#### Highest Degree of Sensitivity

- Detect Changes of 20%

#### Lowest Detection Threshold and Broadest Linear Dynamic Range

- 7-log Range of Gene Expression

#### *StaRT-PCR™* Facilitates Pharmacogenomics

- Accelerates the Application to Biomarkers and Molecular Diagnostics
- Bridges Standardized Data Integration Throughout Drug Development
- Supports FDA New Drug Regulatory Approval Process

for each gene (provided by our proprietary SMIS™). Because of this, it is possible to directly compare the results of any gene expression value to others obtained the same day in the same experiment or at any other time in a separate experiment, even in another laboratory (Table 2). This benefit facilitates inter-laboratory studies, multi-site collaboration and multi-institutional clinical trials, and allows for the development of a pan-organization standardized database for all *StaRT-PCR*™ gene expression values. With standardized results and a standardized database, *StaRT-PCR*™ enables virtually infinite multi-gene (multiplex) analysis in a timeless fashion. Future gene expression measurements can be added to past experimental results. Thus, the data remain alive. In addition, standardized, numeric gene expression values allow for the establishment of “normal” expression levels. Once normal levels are determined, there is no need to run a normal sample comparison in each experiment. This latter benefit is particularly valuable for the development of biomarkers and molecular diagnostic tests based on gene expression.

**Table 2: *StaRT-PCR*™ Data Allows for Inter-Sample and Intra-Sample Comparisons and Renormalization of Values**

	Sample 1	Sample 2
<b>Gene A<sup>1</sup></b> (normalized to ACTB)	210	4800
<b>Gene B<sup>1</sup></b> (normalized to ACTB)	8900	7600
<b>Gene A<sup>2</sup></b> (renormalized to Gene B)	0.02	0.63
<b>Gene B<sup>2</sup></b> (renormalized to Gene B)	1	1

<sup>1</sup> molecules/10<sup>6</sup> ACTB transcripts  
<sup>2</sup> molecules/molecule Gene B transcript  
Values based on triplicate measurements.

### Ruggedness of Methodology

*StaRT-PCR*™ assays meet the ruggedness definition of pharmacogenomics. This is evident by the high degree of reproducibility obtained by the analysis of the same sample under a variety of test conditions such as different laboratories, analysis days, instruments and reagent lots. Thus, *StaRT-PCR*™ is applicable to pharmaceutical development and quality-controlled clinical laboratories. The use of SMIS™ eliminates false PCR negatives and the employment of gene-specific reagents and rigorous standard operating procedures in the SEM Center™ significantly reduce false PCR positives while providing the client with the added value of FDA Good Laboratory Practices (GLP) compliant results.

### Accurate Quantitative Results

*StaRT-PCR*™ provides accurate quantification by providing a standard unit for gene expression values. For each gene, the number of molecules of transcript is normalized to the number of molecules of a reference gene used as a loading control (standardization) by utilizing the constant and stable SMIS™. The target genes and the reference gene are also measured relative to their gene-specific internal standards, which are included in the same SMIS™. Each cDNA sample is diluted until the number of reference gene transcripts it contains is in balance with an aliquot of SMIS™ containing a known number of molecules of the internal standard for that reference gene. This balanced amount of cDNA is then included in each assay along with an aliquot of SMIS™ containing a known number of molecules of internal standard for each transcript. Gene-specific primers are employed to ensure the best possible gene specificity. By controlling each reverse transcriptase (RT) PCR reaction for all known sources of variation and by employing stable, constant standards, these procedures enable precise and accurate calculation of transcript abundance for each gene. In addition, these features enable

**Table 3: Comparison Between *StaRT-PCR*™ Absolute Expression Values and Relative Expression Values**

<i>StaRT-PCR</i> ™ Gene Expression Values	Relative Gene Expression Values
Internal standard included in each assay	No standards
Units in molecules of transcript normalized to a reference	Units in “fold-difference” between samples
Data obtained are alive	Data obtained are only relative between samples measured
Data are independent values and can be mathematically converted to gene expression indices	Data for each gene is dependent on the sample used for comparison

renormalization to any gene measured in the sample (Table 2). This makes *StaRT-PCR*™ data much more valuable than that obtained by other methodologies.

Two general categories for the measurement of gene expression have emerged in the genomic community. These are relative quantification and true quantification with *StaRT-PCR*™ (Table 3). In relative quantification, the expression level of a gene or set of genes is determined in two paired samples (usually diseased vs. normal or treated vs. control or drug responders and non-responders). The gene expression values are then presented as “fold-differences” of expression between the two samples. This approach allows only for the semi-quantitative measurement of gene expression, and always requires that a control sample be available and measured in parallel with the test sample.

True quantitation of gene expression with *StaRT-PCR*™ is a more rigorous assessment of expression levels (Table 3). This approach requires the independent measurement of a calibrated internal standard with SMIS™. Then, the value of the standard can be used to convert the expression levels of the test samples to a numerical value represented as the number of gene transcript molecules. With traditional qPCR (quantitative PCR), this requires the employment of two external standard curves obtained by evaluating a standard dilution series of both the target gene and the reference gene.

In *StaRT-PCR*™, internal standards are included for each gene expression assay in the form of SMIS™. These SMIS™ allow for the determination of true concentrations of each gene transcript in the assay because the SMIS™ are prepared as one lot with known concentrations and fixed ratios of one internal standard to another. Also, because internal standards for reference genes are included, the gene expression values are easily normalized. The final result is that the gene expression values that are obtained with *StaRT-PCR*™ are designated as number of molecules of measured transcript per number of molecules of reference gene. The resulting benefit is that all *StaRT-PCR*™ gene expression measurements stand alone and are not dependent on values obtained from a control sample (Figure 2). In addition, since each gene expression measurement with *StaRT-PCR*™ is determined using our standardized proprietary SMIS™, all measurements can be compared to each other. These comparisons can be made across genes within a sample, across genes across samples, and across experiments (Figure 2).

gene-specific forward and reverse primers and a gene-specific internal standard for each gene formulated into SMIS™ enables this high reproducibility (Table 4 and Figure 1).

### Higher Sensitivity

In the context of transcript abundance measurement, sensitivity reflects the correlation that is seen between the signal observed and the amount of analyte. Data consistent with 100% sensitivity are observed routinely in our SEM Center™. The increase in signal obtained with *StaRT-PCR*™ is in 1:1 correspondence with the amount of analyte present (1, 6).

With our technology, research results move beyond “fold changes” to more precise, quantitative data. *StaRT-PCR*™ is capable of detecting changes in gene expression levels as small as 20%. In addition, since the values are number of molecules, the expression levels of two different genes (or more) can be evaluated within and between samples (Table 2). In the example shown, it can be seen that Gene A is expressed at a much lower level in Sample 1 than is Gene B, whereas Gene A is

expressed at only about half the level of Gene B in Sample 2. This type of analysis can provide valuable insight into the system under investigation. This is not possible with relative or semi-quantitative gene expression values since such data is represented in “fold-changes” relative to a control or “normal” sample, and any one transcript can only be compared to itself.

### Lowest Detection Threshold and Broadest Linear Dynamic Range

The linear dynamic range of *StaRT-PCR*™ allows for the measurement of gene expression across the entire range of 7 logs of gene expression observed in cells, <10 to 10<sup>7</sup> molecules/10<sup>6</sup> molecules reference gene (Table 2 and Figure 1).

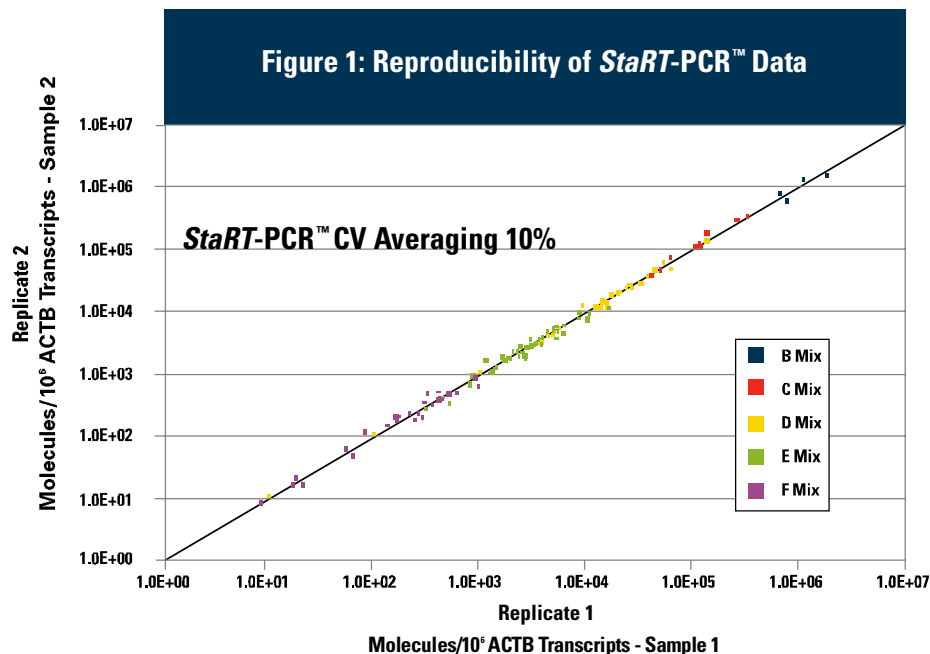
### Accelerates the Application of *StaRT-PCR*™ to Biomarkers and Molecular Diagnostics

*StaRT-PCR*™ enables multi-gene expression profiling. Hundreds of gene expression assays are available (see Web site for details: [www.GeneExpressInc.com](http://www.GeneExpressInc.com)). Because *StaRT-PCR*™ expression values are standardized and numerical, expression values can be compared within and between

Table 4: Metrics of <i>StaRT-PCR</i> ™ Gene Expression Values	
Number of Genes Measured	28
Number of Samples	24
Average CV	10.9%
High Expression Value*	1.5 x 10 <sup>5</sup> (BCL2)
Low Expression Value*	4.0 (ERBB2)
*Number of molecules of transcript per 10 <sup>6</sup> molecules of ACTB transcript. The expression level for each gene was measured in triplicate in each sample.	

### Greater Reproducibility

Replicate data with coefficients of variance (CV) averaging 10% are consistently obtained. The employment of high-throughput, automated robotics, rigorous Standard Operating Procedures (SOPs),



Each data point represents the average of six measurements for one gene (triplicate measurements for Replicate 1 and triplicate measurement for Replicate 2). Each color represents a different mix of internal standard (i.e., Mix D is 600,000 molecules of ACTB and 6000 molecules of other genes as SMIS™, etc). Mix E is 600,000 molecules of ACTB and 600 molecules of other genes as SMIS™, etc). In order to be quantitative, the native template amplicon peak area must be within a 10-fold range of the internal standard amplicon peak area. Each mix provides data over a 100-fold range, because the native template can be anywhere from 1/10 to 10/1 relative to the internal standard. Thus, usable data can be obtained for some genes with more than one internal standard mix, and the entire range of gene expression in cells is covered.

samples (Table 2 and Figure 2). Expression values can be normalized to any other gene measured in all samples, and they can be combined into highly informative biomarkers or Interactive Gene Expression Indices<sup>™</sup> (IGEI<sup>™</sup>) (4, 5).

Since the response of each individual gene is likely to vary from one individual to another, the combination of several genes into IGEI<sup>™</sup> biomarkers serves as a better diagnostic discriminator than the expression value of an individual gene. Multiple genes involved in a disease pathway, required for normal, healthy tissue or as putative targets for a therapeutic drug, may be measured simultaneously using *StaRT-PCR*<sup>™</sup>. Since *StaRT-PCR*<sup>™</sup> data is standardized, the numerical values can be mathematically converted into IGEI<sup>™</sup> values. IGEI<sup>™</sup> may be formulated by combining genes that increase or decrease in expression associated with that disease. These IGEI<sup>™</sup> biomarkers or molecular diagnostics also provide better information regarding inter-individual response to treatment and mechanism of drug action (4, 5).

### Facilitates Pharmacogenomics by Bridging Standardized Data Integration Throughout Drug Development

Gene Express, Inc., provides a way to increase the quality and value of pharmacogenomic data. To put this into perspective, almost all of our clients are using microarrays to identify significant genes in disease pathways and therapeutic areas based on their expression levels. Clients are then using *StaRT-PCR*<sup>™</sup> to look at all of their genes of interest simultaneously and quantitatively. This provides a better understanding of how these genes are interacting to influence disease states, drug responses, etc., and allows for the identification of biomarkers. The *StaRT-PCR*<sup>™</sup> data that they obtain can then be added to a growing standardized database. In addition, *StaRT-PCR*<sup>™</sup> data from multiple genes can be caused to interact mathematically to discover discriminatory biomarker indices for the development of

new drugs and molecular diagnostics.

A synergistic increase in knowledge can be gained with the development of a standardized gene expression database. A common language for gene expression measurement with *StaRT-PCR*<sup>™</sup> enables a standardized expression database annotated with respect to disease state, drug response, therapeutic response, etc. Such a *StaRT-PCR*<sup>™</sup> reference database for biomarkers or clinical diagnostics will allow data collected over time to be compared. All *StaRT-PCR*<sup>™</sup> data may be compared across multiple laboratories and in multi-institutional clinical trials. In addition, the database will allow the comparison of expression data among different functional groups within an organization: preclinical, across all phases of clinical trials and multi-site clinical trials. This will facilitate the stratification of clinical trials based on quantitative, multi-gene expression profiling through IGEI<sup>™</sup> values.

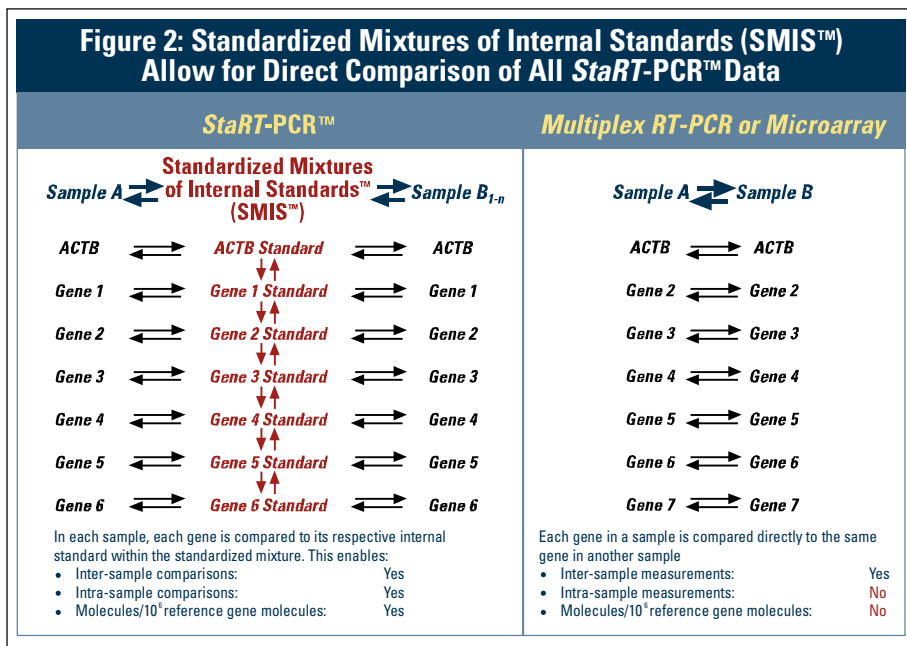
Use of quality-controlled and standardized data increases the value of pharmacogenomics data submitted to the FDA regulatory agencies for new drug approval. This improves cost efficiency by stratifying patients in all phases of clinical trials based on standardized, quality-controlled and multi-gene expression profiling data while

making it easier to identify responders vs. nonresponders.

Our clients who are developing a standardized gene expression database within their companies are positioning themselves to better understand systems biology by relating proteomics and metabolomics data to the standardized gene expression database to elucidate complex disease pathways. *StaRT-PCR*<sup>™</sup> is more than just a technology platform. It is the next-generation, multi-gene transcript abundance analysis system to enhance the understanding of systems biology, the identification of biomarkers and the development of molecular diagnostics. The *StaRT-PCR*<sup>™</sup> data accumulated will enrich drug discovery pipelines by synergistically increasing knowledge across all functional areas of biology across organizations throughout time.

### *StaRT-PCR*<sup>™</sup> Supports Pharmacogenomics for the FDA New Drug Regulatory Approval Process

There is increasing encouragement from the FDA to have effective quality control in place for pharmacogenomic data that is submitted with New Drug Applications (NDA). Since results are standardized and reproducible with integrated quality control, *StaRT-PCR*<sup>™</sup> gene expression data can be



used throughout the R&D process from the early stages of drug target screening, lead identification and validation through preclinical to clinical trials and treatment. The data remain alive and the results obtained during all R&D phases can be directly compared with those obtained elsewhere.

Gene Express, Inc., is getting attention from biopharmaceutical companies, not only because our next-generation transcript profiling technology supports the new FDA regulatory approval process by enabling quality control and standardization, but also because data generated with our platform can be entered into a standardized expression database and enables data integration from different functional groups within an organization. Each group can directly compare expression data across departments. For example, a cancer biology group can compare new data to toxicology data that was captured five years earlier in the drug development process. Also, as discussed earlier, data across institutions (such as for multi-site clinical trials) can be compared.

*StaRT-PCR*<sup>™</sup> data is playing a role in novel, better and more-targeted drug development with higher efficacy and fewer side effects. Biomarkers identified during drug development are easily converted to molecular diagnostics to support patient segmentation (by genetic make-up) during clinical trials and treatment. These properties provide improved cost efficiencies, faster results and support for the FDA new drug regulatory approval process.

Recently, the FDA posted a draft report in which they describe responses to questions regarding quality control issues that must be addressed for multiplex gene expression data submitted in support of drug or molecular diagnostic test development (<http://www.fda.gov/cdrh/oivd/guidance/1210.pdf>). Importantly, all of these issues are effectively addressed with *StaRT-PCR*<sup>™</sup>, but with no other gene expression method (neither microarrays nor real-time RT-PCR).

### **Making the Benefits of *StaRT-PCR*<sup>™</sup> and the SEM Center<sup>™</sup> Work for You Today**

The combination of all of the benefits discussed above make *StaRT-PCR*<sup>™</sup> particularly suited for the needs of genomics research, pharmacogenomics, toxigenomics, molecular diagnostics, and biomarker discovery and assessment (1, 2, 3). For greater convenience and easier access to the technology, Gene Express, Inc., now offers a multi-gene expression analysis service based on *StaRT-PCR*<sup>™</sup>. Gene Express, Inc., provides high throughput, GLP-compliant results at the SEM Center<sup>™</sup>. To find out how *StaRT-PCR*<sup>™</sup> can enhance the quality, reproducibility and standardized value of your gene expression measurements, call Gene Express or visit our Web site today.

### **References**

1. Willey, J.C., E.L. Crawford, C.M. Jackson, D.A. Weaver, J.C. Hoban, S.A. Khuder, & J.P. DeMuth. 1998. Expression measurement of many genes simultaneously by quantitative RT-PCR using standardized mixtures of competitive templates. *Am. J. Respir. Cell Mol. Biol.* 19:6-17.
2. Crawford, E.L., K.A. Warner, S.A. Khuder, R.J. Zahorchak, & J.C. Willey. 2002. Multiplex standardized RT-PCR for expression analysis of many genes in small samples. *Biochem. Biophys. Res. Commun.* 293:509-516.
3. Apostolakis M.J., W.H. Schuermann, M.W. Frampton, M.J. Utell, & J.C. Willey. 1993. Measurement of gene expression by multiplex competitive polymerase chain reaction. *Anal Biochem.* 213:277-284.
4. DeMuth, J.P., C.M. Jackson, D.A. Weaver, E.L. Crawford, D.S. Durzinsky, S.J. Durham, A. Zaher, E.R. Phillips, S.A. Khuder, & J.C. Willey. 1998. The gene expression index c-MYC x E2F-1/p21 is highly predictive of malignant phenotype in human bronchial epithelial cells. *A. J. Respir. Cell Mol. Biol.* 19:18-24.
5. Warner, K.A., E.L. Crawford, A. Zaher, R.J. Coombs, H. Elsalamoty, S.L. Roshong-Denk, I. Sharief, G.V. Amurao, Y. Yoon, A.Y. Al-Astal, R.A. Assaly, D.A. Hernandez, T.G. Graves, C.R. Knight, M.W. Harr, T.B. Sheridan, J.P. DeMuth, R.J. Zahorchak, J.R. Hammersley, D.E. Olson, S.J. Durham, & J.C. Willey. 2003. The c-MYC x E2F-1/p21 interactive gene expression index augments cytomorphologic diagnosis of lung cancer in fine-needle aspirate specimens. *J. Mol. Diagn.* 5:176-83.
6. Crawford, E.L., D.A. Weaver, J.P. DeMuth, C.M. Jackson, S.A. Khuder, M.W. Frampton, M.J. Utell, W.G. Thilley, & J.C. Willey. 1998. Measurement of cytochrome P450 2A6 and 2E1 gene expression in primary human bronchial epithelial cells. *Carcinogenesis.* 19:1867-1871.

**Gene Express, Inc.**, founded in 1992, is a commercial stage genomic biotechnology company that sells next-generation, patented, quantitative, standardized multi-gene expression platform technology (*StaRT-PCR*<sup>™</sup>). Gene Express sells gene assays and biomarkers to assist pharmaceutical and biotech companies with new drug development; clinical diagnostic companies with innovative and patented, molecular diagnostic tests for cancers, infectious diseases, and other therapeutic areas; academic institutions with genomic research; and the US Departments of Defense (DOD) and Homeland Security with biohazardous materials. Gene Express provides multi-gene expression analysis services at its Standardized Expression Measurement (SEM) Center<sup>™</sup> on high throughput automated equipment to researchers in the US, Europe and Japan. Gene Express also designs, produces and markets new gene assay and biomarker system products that allow standardized, reproducible, cost effective measurement of multi-gene activity. Assays for multi-gene expression analysis can be ordered today. Please contact our Business Development Team at 800-820-8341 to find out how Gene Express can add value to your organization.