

StaRT-PCR[™] – The Choice for Defining, Validating and Measuring Biomarkers or Interactive Gene Expression Indices[™] (IGEs[™])

The aftermath of the human genome sequencing project has provided the medical community the opportunity to enhance the ability to detect and treat diseases. With the successful determination of the complete human genome sequence, the groundwork is set for identifying all genes present and their corresponding functions. This is enormous progress. The resultant information will be used to develop more

they represent a physiological snapshot of gene expression values by interacting genes. Thus, *StaRT*-PCR[™] is the premier technology for defining and measuring biomarkers relevant to pharmacogenomics and molecular diagnostics (3).

Molecular Diagnostics:

Genetic Diseases and Their Detection

The fastest growing area of in vitro diagnostics is molecular diagnostics. Early diagnostic tests for genetic diseases involved cytogenetics, the microscopic examination of the chromosomes to identify abnormalities. One of the earliest genetic tests applied to the diagnosis of a genetic disease is the cytogenetic test for Down's Syndrome (trisomy 21). Trisomy 21 is characterized by the appearance of an extra copy of chromosome 21. The cytogenetic test for this disease is to examine the chromosomes through a microscope and count the number of each chromosome. An extra copy of chromosome 21 indicates the presence of Down's Syndrome.

Over time, it has become obvious that there are many diseases that have a genetic basis. The ultimate cause for each specific syndrome is unique but, in general, it is a variation of a specific gene or set of genes that results in the disease phenotype.

The best method used to detect any one genetic disease depends on the nature of the defect. In some cases, it is best to analyze directly the specific DNA segment affected using conventional cytogenetic techniques. Unfortunately, most genetic diseases cannot be detected through the application of classical cytogenetics. This is because the specific alterations of the DNA may be very minute, affecting only a single base or a few bases of the DNA chain. However, a minor alteration in one gene may profoundly affect the expression pattern of many genes. Thus, there has been a concerted effort to develop

methods to measure gene expression patterns (also referred to as gene expression profiling or transcript profiling).

***StaRT*-PCR[™] Measures Gene Expression Patterns**

It is critical that the technologies used to determine gene expression patterns be precise and the data must be of the best quality possible. To meet this challenge, Gene Express, Inc. has developed *StaRT*-PCR[™].

StaRT-PCR[™] is a quantitative, standardized multi-gene expression method with high reproducibility (1, 2, 3). *StaRT*-PCR[™] has the ability to measure gene expression of multiple genes over the entire 7 log range of expression levels observed in cells (1, 2, 3). *StaRT*-PCR[™] also provides standardized, numerical gene expression values allowing for the data to remain alive, be directly compared to other gene expression data, and be manipulated mathematically. In addition, the use of Standardized Mixtures of Internal Standards[™] (SMIS[™]) provides for integrated quality control. All of these attributes makes *StaRT*-PCR[™] the ideal method for gene expression measurement (3).

Standardized, Reproducibility and Numerical Values Allow for Calculation of Interactive Gene Expression Indices[™]

It is believed that most genetically based diseases, including many cancers, are not the result of a single mutation in a single gene. Rather, it is likely most phenotypes (both normal and diseased) will be associated with a particular pattern of expression of more than one gene. If the expression pattern for the relevant set of genes is known in normal cells and diseased cells and if this pattern can be measured, then a molecular diagnostic test is possible. For this reason, many scientists are delineating the expression pattern of many genes in a variety of normal and diseased tissues to identify the relevant sets of "signature" genes that play an

Identifying IGEs[™] Using *StaRT*-PCR[™] Gene Expression Values

- Define a set of putative genetic biomarkers
- Obtain quantitative, standardized, numerical expression levels of each gene in affected and normal tissue or experimental and control samples using *StaRT*-PCR[™]
- Evaluate the expression data empirically using the machine learning software method to determine the rule that discriminates best between affected and normal (experimental and control)
- Validate rule(s) on blinded samples

effective drugs through pharmacogenomics and more accurate early diagnostic tests by integrating biomarkers into molecular diagnostics. Already, there are molecular diagnostic tests for a number of diseases. It is becoming clear that, although some genetic diseases are caused by or can be detected through a single mutation in a single gene, many diseases are due to aberrant function of a group of genes. If measured through *StaRT*-PCR[™], (**S**tandardized **R**everse **T**ranscription **P**olymerase **C**hain **R**eaction) the expression levels of relevant groups of genes can be combined into Interactive Gene Expression Indices[™] (IGEs[™]). These IGEs[™] are much more informative than single gene values since

important role in exhibiting physiological normal and pathophysiological phenotypes.

StaRT-PCR™ is readily applied to multi-gene expression analysis. Also, *StaRT-PCR*™ gene expression data is numerical (number of molecules of transcript is normalized to 10⁶ molecules of reference gene), so the values can be mathematically manipulated and can be presented as Interactive Gene Expression Indices™. It has been shown that IGEIs™ have better diagnostic sensitivities and specificities than do individual gene expression values (4, 5). It is possible to evaluate numerical gene expression values obtained with *StaRT-PCR*™ empirically to determine which genes are the ideal biomarkers and which mathematical relationships of the expression values define the best IGEI™.

More recently, we have developed a working relationship with Genetics Squared, LLC. Genetics² has developed unique analytic tools for the growing bioinformatics market. Through the use of the Genetics² proprietary data analysis system, optimal IGEIs™ can be identified from any set of *StaRT-PCR*™ gene expression data.

A Practical Example of Identifying and Using IGEIs™

One of the key ingredients to defining useful IGEIs™ is the access to numerical gene expression data. *StaRT-PCR*™ provides quantitative, standardized, numerical gene expression values that can be used to generate IGEIs™. The technology has been used in a number of laboratories to obtain gene expression profiles and to relate these profiles to phenotypes (3, 4, 5).

DeMuth and colleagues published a peer-reviewed study exemplifying how *StaRT-PCR*™ data can be used to test hypotheses regarding the correlation between gene expression and phenotype and to define IGEIs™ (3). These authors hypothesized that the expression pattern of a set of genes could be associated with the malignant phenotype of human bronchial epithelial cells. They first identified 15 genes that were involved in cell cycling and, thus, may also be associated with malignancy. The expression levels of these 15 genes were then determined by *StaRT-PCR*™. Analysis of the gene expression data indicated that the expression level of no single gene was associated with the malignant phenotype. However, by combining the expression levels

of those genes that were generally altered in malignant tissue relative to control tissue and testing a number of mathematical relationships, it was determined that the IGEI™ of *c-myc* x *E2F1/p21* could best discriminate between malignant and normal tissue. These studies have been extended to fine needle aspirate samples of a larger population with excellent diagnostic sensitivity and specificity (6). Currently, this test is in larger validation multicenter study.

Summary

Through pharmacogenomics and molecular diagnostics, there lies promise for individualized and more effective drugs and early diagnostic tests. The determination of gene expression patterns will provide information to associate transcript profiles with phenotypes. *StaRT-PCR*™ provides the robust, quality controlled data that is required for the development of dependable tests based on multi-gene expression measurement (7, 8, 9). *StaRT-PCR*™ data can be combined into Interactive Gene Expression Indices™ that are highly informative and applicable to pharma-cogenomics and the development of molecular diagnostics.

References

1. Apostolakos, M.J., W.H. 1. 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.
2. 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.
3. Willey, J.C. 2004. Quality-Controlled Multi-Gene Expression Measurement, *PharmaGenomics.* **4**:21-33.
4. 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.
5. 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. *Am. J. Respir. Cell Mol. Biol.* **19**:18-24.
6. 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.
7. Crawford, E.L., G.J. Peters, P. Noordhuis, M.G. Rots, M. Vondracek, R.C. Grafstrom, K. Lieuallen, G. Lennon, R.J. Zahorchak, M.J. Georgeson, A. Wali, J.F. Lechner, P.S. Fan, M.B. Kahaleh, S.A. Khuder, K.A. Warner, D.A. Weaver, & J.C. Willey. 2001. Reproducible gene expression measurement among multiple laboratories obtained in a blinded study using standardized RT (StaRT)-PCR. *J Mol Diagn.* **6**:217-225.
8. Crawford, E.L., S.A. Khuder, S.J. Durham, M. Frampton, M. Utell, W.G. Thilly, D.A. Weaver, W.J. Ferencak, C.A. Jennings, J.R. Hammersley, D.A. Olse, & J.C. Willey. 2000. Normal bronchial epithelial cell expression of glutathione transferase P1, glutathione transferase M3, and glutathione peroxidase is low in subjects with bronchogenic carcinoma. *Cancer Research.* **60**:1609-1618.
9. Willey, J.C., E.L. Coy, M.W. Frampton, A. Torres, M. J. Apostolakos, G. Hoehn, W.H. Schuermann, W.G. Thilly, D.E. Olson, J.R., Hammersley, C.L. Crespi, & M.J. Utell. 1997. Quantitative RT-PCR measurement of cytochromes p450 1A1, 1B1, and 2B7, microsomal epoxide hydrolase, and NADPH oxidoreductase expression in lung cells of smokers and nonsmokers. *Am. J. Respir. Cell Mol. Biol.* **17**:114-124.