Abstract: This paper evaluates the performance of the newest technique for monitoring the expression of a panel of pathway- or disease-specific genes: the RT² Profiler PCR Array System from SA Biosciences. The PCR Array System combines the quantitative performance of SYBR® Green-based real-time PCR with the multiple gene profiling capabilities of a microarray. The PCR Array is a 96- or 384-well plate containing RT² qPCR Primer Assays for a set of 84 related genes, plus five housekeeping genes, and three controls. A complete system includes an instrument-specific master mix and an optimized first strand synthesis kit. This paper presents scientific data showing that PCR Arrays have the sensitivity, reproducibility, and specificity expected from real-time PCR techniques. As a result, this technology brings focused gene expression profiling to any biological laboratory setting with a real-time PCR instrument.

Introduction

The RT² Profiler PCR Array System is the most reliable and accurate tool for analyzing the expression of a focused panel of genes using SYBR Green-based real-time PCR. It brings together the quantitative performance of real-time PCR and the multiple gene profiling capability of microarrays. Each PCR Array profiles the expression of 84 genes relevant to a specific pathway or disease state. Expression levels are measured by gene-specific RT² qPCR Primer Assays optimized for simultaneous use in the PCR Array System.

RT² qPCR Primer Assays are key components in the PCR Array System. Each qPCR assay on the array is uniquely designed for use in SYBR Green real-time PCR analysis. The assay design criteria ensure that each qPCR reaction will generate single, gene-specific amplicons and prevent the co-amplification of non-specific products. The qPCR Assays used in PCR Arrays are optimized to work under standard conditions enabling a large number of genes to be assayed simultaneously. Their specificity is guaranteed by SA Biosciences when RT² SYBR Green qPCR Master Mixes are used as part of the complete PCR Array System protocol.

The RT² Profiler PCR Array System is specifically designed to meet the unique challenges of profiling pathway-focused sets of genes using real-time PCR. Simultaneous gene expression analyses require similar qPCR efficiencies for accurate comparison among genes. RT² qPCR Primer Assays are designed with an amplicon size ranging from 100 to 250 bp and with PCR efficiencies uniformly greater than 90%. Overall, more than 10 thermodynamic criteria are included in the design of each RT² qPCR Primer Assay to ensure the most reliable and accurate results for pathway-based gene expression analysis in the PCR Array System.
Experimental Protocol

Figure 1 depicts an overview of the PCR Array procedure. The protocol takes only two hours to perform (per sample) from start to finish. Start by converting the experimental RNA samples into PCR template with the RT² First Strand Kit. Then, combine the template with an instrument-specific and ready-to-use RT² SYBR Green qPCR Master Mix. Add equal aliquots of this mixture (25 μl for 96-well or 10 μl for 384-well plates) to each well of the same PCR Array plate containing the pre-dispensed gene-specific primer sets, and perform PCR. Use your instrument’s software to calculate the threshold cycle (Ct) values for all the genes on each PCR Array. Finally, calculate fold-changes in gene expression for pair-wise comparison using the ∆∆Ct method. A quick examination of Ct value consistency for the housekeeping genes quickly indicates the proper normalization method. A similarly quick evaluation of the built-in RNA quality controls elements provides the relative levels of genomic DNA contamination and inhibitors of either the reverse transcription or the PCR itself.

How It Works

<table>
<thead>
<tr>
<th>How It Works</th>
<th>PCR Array Design and Gene Content</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Isolate RNA from your experimental samples.</strong></td>
<td>Each RT² Profiler PCR Array contains gene-specific qPCR assays for a thoroughly researched set of 84 genes relevant to a pathway or disease state and three RNA quality control elements. (See Figure 2 for the layout of a typical PCR Array). Researchers are able to focus on genes related to their biological pathway or disease state with our pre-designed pathway- or application-specific gene panels. By limiting the range to less than one hundred genes (instead of thousands at one time), analysis can be achieved much faster and with greater precision due to the highly specific, yet smaller amount of data to analyze. As a result, more meaningful data can be obtained in less time. This process also streamlines the preparation stages of the experiment because the relevant genes are already grouped into one ready-to-use assay.</td>
</tr>
<tr>
<td>Start with as little as 25 ng of total RNA (1 μg is recommended). Treat with DNase.</td>
<td></td>
</tr>
<tr>
<td><strong>Prepare cDNA from your RNA samples.</strong></td>
<td><strong>Figure 2: Layout of the Cataloged PCR Arrays</strong></td>
</tr>
<tr>
<td>PCR Arrays are designed for use with the RT² First Strand Kit.</td>
<td>Wells A1 through G12 contain individual qPCR assays for 84 genes relevant to a biological pathway or disease state. Wells H1 through H5 contain a panel of housekeeping genes (HK1-HK5) used for normalizing the PCR Array data. Well H6 contains a Genomic DNA Control (GDC) primer set that specifically detects non-transcribed, repetitive genomic DNA with a high level of sensitivity. Wells H7 through H9 contain replicate Reverse Transcription Controls (RTC) used to verify the efficiency of the RT reaction with a qPCR assay that specifically detects template synthesized from the first strand synthesis kit’s built-in external RNA control. The replicate Positive PCR Controls (PPC) in wells H10 through H12 report on the efficiency of the polymerase chain reaction itself. These elements use a pre-dispensed artificial DNA sequence and the primer set that detects it. The two sets of replicate control wells (RTC and PPC) also test for inter-well and intra-plate consistency.</td>
</tr>
<tr>
<td><strong>Add cDNA to RT² qPCR Master Mix.</strong></td>
<td><strong>PCR Array Benefits</strong></td>
</tr>
<tr>
<td>RT² SYBR Green qPCR Master Mixes add guaranteed performance.</td>
<td>- <strong>Pathway-Focused:</strong> Efficiently profiles the expression of a large panel of genes relevant to a pathway or disease state</td>
</tr>
<tr>
<td><strong>Aliquot the Mixture Across Your PCR Arrays.</strong></td>
<td>- <strong>Simple and Accurate:</strong> Easy-to-use qRT-PCR based procedure provides high performance levels</td>
</tr>
<tr>
<td>Each PCR Array profiles the expression of 84 pathway-specific genes plus controls.</td>
<td>- <strong>Easy Access:</strong> Brings the power of expression profiling to any lab with real-time PCR capabilities</td>
</tr>
<tr>
<td><strong>Perform Thermal Cycling</strong></td>
<td></td>
</tr>
<tr>
<td>Collect real-time amplification data (Ct values) using your instrument’s software.</td>
<td></td>
</tr>
<tr>
<td><strong>Analyze Fold Changes in Expression.</strong></td>
<td></td>
</tr>
<tr>
<td>Simply cut-and-paste the Ct values collected by your real-time PCR instrument into the PCR Array analysis spreadsheet.</td>
<td></td>
</tr>
</tbody>
</table>

Figure 1: The Complete PCR Array Procedure is Easy-to-use and Requires Minimal Hands-on Time.
Pathway-Focused PCR Arrays

The 96- or 384-well format of the RT² Profiler PCR Arrays is uniquely suited to SABiosciences’ pathway-focused design concept. This product line combines the current understanding of important biological pathways with real-time PCR technology to generate application-specific research tools. To compile each product’s comprehensive list of genes and to continually expand the breadth of available products, a systematic process comprised of literature surveys, database searches, expert review, and user feedback is utilized. SABiosciences now has the largest collection of pathway and biological pathways with real-time PCR technology to generate product line combines the current understanding of important suited to SABiosciences’ pathway-focused design concept. This pre-designed application-specific PCR Arrays accelerate, simplify, and improve life science research by saving time, effort, and resources. Currently, PCR Arrays are available for many pathways including apoptosis, inflammation, signal transduction, cancer and other diseases. Visit the SABiosciences web site (www.SABiosciences.com) for a complete list.

Table 1: Examples of Cataloged Pathway-Focused PCR Arrays

<table>
<thead>
<tr>
<th>Research Application</th>
<th>PCR Array Example</th>
<th>Gene Content Selected for the PCR Array Example</th>
</tr>
</thead>
<tbody>
<tr>
<td>Biological Process</td>
<td></td>
<td></td>
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<tr>
<td></td>
<td>Human Apoptosis</td>
<td>TNF Ligands and their Receptors</td>
</tr>
<tr>
<td></td>
<td></td>
<td>BCL2 Family Members</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Caspasans</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Death and Effectors Domains</td>
</tr>
<tr>
<td></td>
<td></td>
<td>ATM and p53 Pathways</td>
</tr>
<tr>
<td>Functionally or Structurally Related Genes</td>
<td>Mouse Common Cytokines</td>
<td>Interferons and Interleukins</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Bone Morphogenetic Proteins</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Tumor Necrosis Factors</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Other Various Growth Factors</td>
</tr>
<tr>
<td>Signal Transduction Pathways</td>
<td>Human NF-κB Signaling Pathway</td>
<td>Extracellular Ligands and Receptors</td>
</tr>
<tr>
<td></td>
<td></td>
<td>NF-κB and IκB Family Members</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Kinases</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Transcription Factors</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Responsive Genes</td>
</tr>
<tr>
<td>Disease</td>
<td>Human Cancer PathwayFinder™</td>
<td>Cell Cycle Control and DNA Damage Repair</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Apoptosis and Cell Senescence</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Cell Adhesion</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Angiogenesis</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Invasion and Tumor Metastasis</td>
</tr>
</tbody>
</table>

Customized PCR Arrays

For researchers who have special gene expression profiling needs, SABiosciences offers a streamlined custom design and array production service. SABiosciences’ Custom PCR Arrays provide researchers the flexibility to 1) validate a focused panel of genes identified by a high-density, genome-wide microarray, 2) modify the gene content of an existing PCR Array to better fit their research project, or 3) characterize a pathway or otherwise focused gene panel not covered by one of the cataloged PCR Arrays. The content of a PCR Array may also be subdivided into multiple sets of a smaller number of gene targets. This format allows for the characterization of multiple biological or technical replicates on the same array and during the same run. Like the cataloged products, Custom PCR Arrays are also available in either 96- or 384-well plate formats.

The Complete PCR Array System

The complete PCR Array System includes the RT² Profiler PCR Arrays, the RT² SYBR Green qPCR Master Mixes and the RT² First Strand Kit. These system components are optimized for SYBR Green real-time PCR detection. The primer design and the optimized master mix formulation work together to insure the specificity of each assay in the array. The instrument-specific PCR Array plate formats and the master mixes containing the appropriate reference dyes also provide the PCR Arrays with the flexibility to match most real-time PCR platforms. The RT² First Strand Kit provides superior sensitivity and an External RNA Control detected by the PCR Array that helps test the quality of the input RNA material.

Why the PCR Array System Works: Component 1: RT² qPCR Primer Assays

The greatest challenge for the PCR Array system is the amplification of every relevant, pathway- or disease-focused gene during the same run. The same uniform PCR conditions must be used while still achieving the high level of sensitivity, specificity, and reproducibility expected of real-time PCR. SABiosciences has designed the best possible qPCR assays and optimized the PCR master mix formulation for SYBR Green detection by experimentally testing thousands of qPCR assays under many reaction conditions.

RT² qPCR Primer Assays: Key Primer Design Criteria

Three of the most important primer design criteria in our experimentally verified computer algorithm:

1. **Specificity:** Using BLAST and other algorithms, the specificity of each primer set is measured against the entire human, mouse, or rat genome to prevent the amplification of sequence-related, non-specific secondary products. The primer specificity is also checked against the E. coli genome to assure that the primers do not amplify bacterial genomic DNA, a common but minor contaminant of many Taq DNA polymerases.

2. **Uniformity:** To use the same annealing temperature for every well in each PCR Array, only primer pairs with similar GC contents, melting temperature ($T_m$), and other chemical and physical properties are used.

3. **Efficiency:** Short amplicons (~100 to 200 bp) have been chosen for our primer pairs so that the enzyme replicates the entire sequence in the time allotted by the cycling program. Several filters are also used to strengthen the 3-prime anchoring of the primers, eliminating the amplification of dimers and other non-specific annealing events.

Email support@SABiosciences.com
Web www.SABiosciences.com
Why the PCR Array System Works: Component 2: RT² qPCR Master Mixes

PCR master mix quality also plays an important role in the performance of SYBR Green-based real-time PCR. A tightly controlled hot-start Taq DNA polymerase is a critical component for success. The RT² qPCR Master Mixes from SABiosciences utilize a unique and proprietary chemically-modified HotStart Taq polymerase which only gains full activity after its heat activation step. Under these conditions, non-specific priming events occurring at low temperatures are not extended. Other master mixes often amplify the resulting templates into non-specific products which can cause false positive results. In addition, the RT² qPCR Master Mixes include proprietary chemical components that further minimize primer dimer formation and ensure high amplification efficiencies for even the most difficult to amplify genes. The combination of the RT² qPCR Primer Assay design and the high performance of the RT² SYBR Green qPCR Master Mix formulation is the foundation for the guaranteed specificity of the assays on the PCR Array.

Why the PCR Array System Works: Component 3: RT² First Strand Kit

The RT² First Strand Kit contains all of the reagents needed not only to convert RNA into first strand cDNA, but also for the removal of genomic DNA from the RNA in the simple two-step 30-minute reaction. A proprietary genomic DNA elimination buffer completely removes any residual genomic DNA from your RNA sample. Then, the optimized formulation also allows you to directly use the RNA preparation for reverse transcription and finally real-time PCR without affecting reaction performance. By eliminating genomic DNA contamination, real-time PCR signal intensities accurately reflect the relative level of gene-specific mRNA transcript.

The kit also includes a built-in External RNA Control, an in vitro transcript with an artificial sequence designed to help test for inhibitors of reverse transcription. The Reverse Transcription Control (RTC) in the PCR Array specifically detects cDNA template generated by the kit from the external RNA control. A reproducible threshold cycle value from this control indicates a consistent and high level of RNA quality and transcription efficiency. Such a result provides a greater degree of confidence in the final results.

The RT² First Strand Kit is optimized for use with the RT² SYBR Green qPCR Master Mixes and subsequent gene expression analysis with the RT² Profiler PCR Arrays as part of the complete PCR Array System. Random hexamers and oligo-dT prime reverse transcription in an unbiased manner and capture more difficult-to-detect genes. The reverse transcriptase, optimized magnesium concentration, and other buffer components maximize cDNA product yield and length. The RT² First Strand Kit contains a complete set of reagents for the conversion of RNA into PCR template and provides greater control over RNA quality than other available kits or enzyme sources. Table 2 summarizes the features of the RT² Profiler PCR Array System.

PCR Array Performance: Sensitivity

Researchers continually attempt to detect genes at ever lower levels of expression and in ever smaller amounts of total RNA. To meet these needs, the PCR Array System must pass a very stringent test of sensitivity. A wide variety of universal RNA amounts were characterized with the PCR Array System and an array representing inflammatory cytokine and receptor genes that are known to be expressed at very low levels. Figure 3 plots the percent positive call (the percentage of genes with Ct < 35) versus the amount of input RNA. The results indicate that the PCR Array System achieves greater than 80 percent positive calls with input total RNA amounts as low as 25.0 ng and as high as 1.0 μg (or even 5.0 μg) per array plate. For other pathways or gene panels expressed at higher levels, the sensitivity of the system may be further improved, potentially yielding high positive call rates with even lower amounts of input total RNA. However, the recommended amount of input RNA for first-time users is 0.5 to 1.0 μg to assure a maximum number of positive calls. The minimum recommended RNA amount is 25.0 ng, because the percent positive call drops significantly with less material.

Table 2: Features of Complete RT² Profiler PCR Array System

<table>
<thead>
<tr>
<th>Array Design</th>
<th>84 pathway-focused genes</th>
</tr>
</thead>
<tbody>
<tr>
<td>1 Genomic DNA control</td>
<td>5 housekeeping genes</td>
</tr>
<tr>
<td>3 Reverse Transcription Controls (RTC)</td>
<td>3 Positive PCR controls (PPC)</td>
</tr>
<tr>
<td>Primer Design</td>
<td>Specificity: Sequence alignment filter</td>
</tr>
<tr>
<td></td>
<td>Uniformity: Consistent melting and annealing temperatures</td>
</tr>
<tr>
<td></td>
<td>Efficiency: Short amplicon sequence</td>
</tr>
<tr>
<td>Master Mix</td>
<td>Instrument-specific SYBR Green formulations</td>
</tr>
<tr>
<td></td>
<td>Supports all ABI, Bio-Rad, MJ Research, and Stratagene platforms</td>
</tr>
<tr>
<td>Hot Start Enzyme</td>
<td>No extension of non-specific priming events</td>
</tr>
<tr>
<td></td>
<td>No amplification of secondary products like primer dimers</td>
</tr>
<tr>
<td>First Strand Synthesis</td>
<td>Optimized gDNA elimination buffer prevents false positive signals</td>
</tr>
<tr>
<td></td>
<td>Built-in External RNA Control to test for inhibitors of RT</td>
</tr>
</tbody>
</table>

Figure 3: High Positive Call Rates with as little as 25 ng of Total RNA

The RT² Profiler PCR Array System yields high positive call rates with as little as 25 ng of total RNA. Different amounts of XpressRef® Human Universal RNA (25, 50, 100, 500, and 1000 ng) were characterized with the Human Inflammatory Cytokines and Receptors PCR Array, the RT² First Strand kit and the RT² SYBR Green/Fluorescein qPCR Master Mix on the Bio-Rad iCycler instrument. The percent positive call rate (the percentage of genes with Ct < 35) is plotted versus the input amount of total RNA.
**PCR Array Performance: Specificity**

The PCR Array System has been designed and optimized for the SYBR Green based detection method used by most real-time systems, making the PCR Array System very flexible and widely applicable. Concerns have been raised over the specificity of SYBR Green-based detection and its ability to amplify only one gene-specific amplicon product, because it detects double-stranded DNA non-specifically. SABiosciences' experimentally verified primer design algorithm, used for the PCR Arrays, guarantees the generation of single, gene-specific amplicons without the co-amplification of primer dimers or other non-specific secondary products.

For an example of a stringent test of PCR Array specificity, we characterized the real-time PCR dissociation curves of each gene on a PCR Array representing highly homologous members of the TGFβ and Bone Morphogenetic Protein (BMP) gene families. Products were also characterized by agarose gel electrophoresis. Figure 4 displays the representative dissociation curves and the agarose gel results for the BMP gene family. Each dissociation curve contains only one peak, and each agarose gel lane contains only one band of the predicted size. The results indicate that the PCR Array amplifies gene-specific products despite the expression of highly homologous members of the same gene family in the same RNA sample. The optimized PCR Array System now brings a level of specificity to SYBR Green-based detection that most thought could be achieved only by more expensive probe-based methods.

**PCR Array Performance: Reproducibility**

The quantitative nature of real-time PCR should impart a high degree of reproducibility onto the PCR Array System. To test this notion, two different end-users characterized, in technical replicates (n = 4), the same universal total RNA sample, each with two separate manufacturing lots of a cataloged PCR Array on two separate days. The raw threshold cycle values for the entire array’s gene panel as determined by each of the first end-user’s replicates versus each of the second end-user’s replicates. Panel A compares the raw threshold cycle values of the array’s gene panel as determined by both of the other two instruments in scatter plots (Panel B) lists the correlation coefficient of the linear curve fit for each scatter plot comparison.

To directly demonstrate that the results from the PCR Array System are indeed reproducible, the fold-differences in the expression of drug metabolism genes between two different RNA samples were compared across three different real-time PCR instrument platforms. In each gene expression profile comparison shown in Figure 6, the curve fit to a straight line with a slope of one (1) has a correlation coefficient of 0.97 or higher. Assuming good RNA sample preparation and proper execution of the PCR Array protocol, any differences observed in gene expression levels are attributable to the biological conditions under study and not experimental variation associated with this level of reproducibility in the technology itself. Table 3 summarizes the typical performance of the RT² Profiler PCR Array.

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**Figure 4: High Specificity with the PCR Array System**

The RT² Profiler PCR Arrays demonstrate a high degree of specificity for their target genes. XpressRef Human Universal Total RNA (5 μg) was characterized on the Human TGFβ / BMP Signaling Pathway PCR Array using the RT² SYBR Green/Fluorescein qPCR Master Mix on the Bio-Rad iCycler instrument. After a standard melting curve program, dissociation curves were obtained (Panel A), and the products were characterized by agarose gel electrophoresis (Panel B).

**Figure 5: High User-to-User Reproducibility**

The PCR Array System demonstrates a high degree of user-to-user reproducibility. Two different end-users characterized template cDNA prepared from Human XpressRef Universal Total RNA (5.0 μg) in technical quadruplicates using the Human Drug Metabolism PCR Array and the RT² SYBR Green / Fluorescein qPCR Master Mix on the Bio-Rad iCycler. Panel A compares the raw threshold cycle values of the array’s gene panel as determined by both of the other two instruments in scatter plots (Panel B) lists the correlation coefficient of the linear curve fit for each scatter plot comparison.

**Table 3: Typical Performance of the RT² Profiler PCR Array**

<table>
<thead>
<tr>
<th>Sensitivity</th>
<th>Dynamic Range</th>
<th>Specificity</th>
<th>Reproducibility</th>
</tr>
</thead>
<tbody>
<tr>
<td>80 % Positive Call with as little as 25 ng</td>
<td>At least five (5) orders of magnitude</td>
<td>Primers amplify single, target-specific PCR products</td>
<td>Correlation coefficients (R) : 0.99 for intra-lab raw C values</td>
</tr>
<tr>
<td>Correlation coefficients (R) : 0.97 for inter-lab fold-change values</td>
<td>Average standard deviation of 0.25 threshold cycles</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

**Figure 6: High Instrument-to-Instrument Reproducibility**

PCR Arrays demonstrate a high degree of instrument-to-instrument reproducibility. Two different MAQC RNA samples were characterized using the Human Drug Metabolism PCR Array and either the RT² SYBR Green / Fluorescein qPCR Master Mix on the Bio-Rad Cyclers or the RT² SYBR Green / ROX qPCR Master Mix on either the Stratagene MX3000p or the ABI 7500 FAST instrumentation. The fold-difference in the expression of the entire array’s gene panel between the two RNA samples determined by each instrument was calculated and compared with both of the other two instruments in scatter plots (Panel A) and the correlation coefficients of the linear curve fits (Panel B).
PCR Array Application Example I: Identifying and Monitoring Oncogenic Pathways

Materials and Methods: Template cDNAs prepared from normal human breast and human breast tumor #1 total RNA (BioChain Institute, Inc., 5.0 μg) were characterized in technical triplicates using the Human Cancer PathwayFinder PCR Array and the RT2 SYBR Green/Fluorescein qPCR Master Mix on the iCycler PCR System.

Triplicate total RNA samples prepared from normal human breast and human breast tumor #2 total RNA (BioChain Institute, Inc., 1.0 μg) were converted into template cDNA and then characterized using the Human Extracellular Matrix and Adhesion Molecules PCR Array and the RT2 SYBR Green/Fluorescein qPCR Master Mix on the iCycler® PCR System.

Results: Gene expression profiling is important for discovering and validating tumor biomarkers and therapeutic targets. Using the Cancer PathwayFinder PCR Array and the Human Extracellular Matrix and Adhesion Molecules PCR Array, we examined the gene expression profiles exhibited by two different human breast tumors relative to normal tissues. The study compared the relative expression of both tumorigenesis- and adhesion-related genes between each tumor sample and a normal breast tissue sample. This study provides an example of the identification of a pathway affected by the transformation of a particular tumor type.

Total RNA samples from normal breast tissue and the first of two unmatched breast tumor were analyzed using the Cancer PathwayFinder PCR Array. This PCR Array includes representative genes from the following biological pathways involved in tumorigenesis: adhesion, angiogenesis, apoptosis, cell cycle control, cell senescence, DNA damage repair, invasion, metastasis, signal transduction molecules, and transcription factors.

Figure 7 displays a scatter plot report of the results from the Cancer PathwayFinder PCR Array experiment, indicating the positions of several noteworthy genes based on their large fold-differences in expression between the normal breast and the breast tumor samples. Of the 84 cancer pathway-focused genes in this array, 24 genes demonstrated at least a 3-fold difference in gene expression between normal breast tissue and the breast tumor. Up-regulation was observed in 17 genes, while 7 genes appeared to be down-regulated in the tumor samples, for a total of 24 differentially regulated genes (Table 4).

A subset of six of the 24 genes (ITGA2, ITGA4, ITGB3, MCAM, MMP9, and TIMP3) represents adhesion and extracellular matrix molecules. ITGB3 was down-regulated, while the other five genes were up-regulated. The results suggest that changes in the expression of genes involved in cellular interactions played an important role in the transformation of this and perhaps other breast tumors. To further test this hypothesis and to analyze the expression of other adhesion-related genes, a second breast tumor sample was characterized using a cellular adhesion-focused PCR Array.

Table 4: Changes in expression for cancer-related genes between normal human breast and human breast tumor #1.

<table>
<thead>
<tr>
<th>Gene</th>
<th>Fold change Tumor/Normal</th>
<th>p value</th>
<th>Average Raw C&lt;sub&gt;t&lt;/sub&gt; (Tumor-Normal)</th>
</tr>
</thead>
<tbody>
<tr>
<td>MMP9</td>
<td>542.45</td>
<td>0.0000</td>
<td>21.8</td>
</tr>
<tr>
<td>TIMP3</td>
<td>39.85</td>
<td>0.0000</td>
<td>30.5</td>
</tr>
<tr>
<td>TNF</td>
<td>27.54</td>
<td>0.0001</td>
<td>31.1</td>
</tr>
<tr>
<td>ITGA4</td>
<td>15.10</td>
<td>0.0000</td>
<td>21.1</td>
</tr>
<tr>
<td>TGFB1</td>
<td>12.27</td>
<td>0.0012</td>
<td>24.6</td>
</tr>
<tr>
<td>BCL2</td>
<td>9.74</td>
<td>0.0030</td>
<td>21.1</td>
</tr>
<tr>
<td>GZMA</td>
<td>9.30</td>
<td>0.0003</td>
<td>25.5</td>
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<tr>
<td>TEK</td>
<td>6.88</td>
<td>0.0003</td>
<td>22.3</td>
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<tr>
<td>JUN</td>
<td>5.34</td>
<td>0.0001</td>
<td>19.9</td>
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<td>APAF1</td>
<td>5.34</td>
<td>0.0018</td>
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<td>ATM</td>
<td>5.34</td>
<td>0.0001</td>
<td>19.9</td>
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<tr>
<td>ITGA2</td>
<td>5.34</td>
<td>0.0042</td>
<td>26.8</td>
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<td>PIK3R1</td>
<td>5.34</td>
<td>0.0001</td>
<td>21.3</td>
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<tr>
<td>SYK</td>
<td>4.65</td>
<td>0.0003</td>
<td>22.5</td>
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<tr>
<td>PLAUR</td>
<td>4.44</td>
<td>0.0007</td>
<td>26.4</td>
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<tr>
<td>MCAM</td>
<td>4.14</td>
<td>0.0000</td>
<td>28.2</td>
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<td>PLAU</td>
<td>3.61</td>
<td>0.0132</td>
<td>27.8</td>
</tr>
<tr>
<td>ETS2</td>
<td>3.61</td>
<td>0.0015</td>
<td>23.5</td>
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<td>ANGPT1</td>
<td>3.36</td>
<td>0.0028</td>
<td>31.3</td>
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<td>3.36</td>
<td>0.0031</td>
<td>24.7</td>
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<td>TERT</td>
<td>3.29</td>
<td>0.0314</td>
<td>34.1</td>
</tr>
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Figure 7: Relative expression comparison for 84 cancer-related genes between normal human breast and human breast tumor #1. The figure depicts a log transformation plot of the relative expression level of each gene (2<sup>-ΔCt</sup>) between breast tumor (x-axis) and normal breast (y-axis). The gray lines indicate a four-fold change in gene expression threshold.

The figure depicts a log transformation plot of the relative expression level of each gene (2<sup>-ΔCt</sup>) between breast tumor (x-axis) and normal breast (y-axis). The gray lines indicate a four-fold change in gene expression threshold.

Table 4: Changes in expression for cancer-related genes between normal human breast and human breast tumor #1. Genes from the experiment in Figure 7 that exhibit a three-fold or greater change in expression between normal and tumor breast tissue are listed.
Total RNA samples from normal breast tissue and the second of the two unmatched breast tumors were characterized on the Extracellular Matrix and Adhesion Molecules PCR Array. Genes that displayed at least a 3-fold difference in expression between the samples are listed in Table 5. On this array, a larger number of genes exhibited differential expression in the second tumor than was observed for the first tumor on the Cancer PathwayFinder PCR Array. A total of 38 genes had a different level of expression in the breast tumor than in the normal breast tissue, with 27 genes showing up-regulation and 11 genes showing down-regulation.

Table 5: Changes in relative expression for genes encoding ECM and adhesion molecules between normal human breast and human breast tumor #2. The table lists genes that exhibit at least a three-fold difference in expression in the breast tumor sample when compared to the normal breast tissue. The raw threshold cycle (Ct) values seen in the two samples are also listed for comparison.

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The first and second breast tumor sample displayed concordant results for four genes (MMP9, TIMP3, ITGA4, and ITGB3) that changed expression in the same direction on the Cancer PathwayFinder PCR Array and the Extracellular Matrix and Adhesion Molecules PCR Array. These results not only further verify that cellular adhesion genes changed their expression in these two particular breast cancer tumors, but also suggest a more general role for these genes in breast tissue transformation.

These types of studies provide a new and convenient way to investigate the mechanisms underlying oncogenesis of specific tumors on a pathway-focused basis. The data shown here is consistent with known principles, that changes in the expression of genes related to cellular adhesion play a role in the transformation of breast tissue1-2. Alterations in the expression of these genes enhance or inhibit metastasis of the tumor from its original location and may aid tumor invasion into a new tissue or organ. A PCR Array focusing on Human Tumor Metastasis is available and could be used to continue this study.
PCR Array Application Example II:
Monitoring Cytokine Expression Levels

Materials and Methods: Peripheral Blood Mononuclear Cells (PBMC) were treated with or without 50 ng/ml PMA + 1 ng/ml ionomycin for 6 or 24 hours. After each incubation period, total RNA was isolated from each preparation, and first strand cDNAs were prepared from 500 ng total RNA of each sample using the RT² First Strand Kit. Template cDNAs were characterized in technical replicates using the Human Common Cytokine PCR Array with the RT SYBR Green/ROX qPCR Master Mix on the 7500 FAST® Real-Time PCR System (Applied Biosystems). Fold changes in gene expression between the stimulated and resting PBMC RNA were calculated using the ΔΔCt method in the PCR Array Data Analysis template.

To validate the results obtained from the PCR Array, the protein level of eight selected cytokines secreted by the PBMC (IL-2, 4, 5, 10, 12, 13, and IFN-γ and TNF-α) was measured. Cell supernatants were collected at different time points (0, 6, 24, and 48 hours) and the cytokines were measured by enzyme-linked immunosorbent assay (ELISA) using the Human Th1 / Th2 Cytokines Multi-Analyte Profiler ELISAArray™ Kit. Optical Density (OD) readings for each protein analyte from the samples were compared to a standard curve for quantification of the amount of protein in the original samples.

Results: Cytokine quantification is an important element in studies of inflammation and immune responses. Quantitative RT-PCR, a rapid and sensitive assay, is the preferred method to quantify cytokine mRNA levels because they are often expressed at low levels. The PCR Array System offers a simple, reliable and sensitive tool for multiple cytokine profiling. Using the Human Cytokine PCR Array, we have monitored the mRNA levels of 84 different cytokines in stimulated versus and untreated human peripheral blood mononuclear cells (PBMC).

The gene expression results identify 23 up-regulated and 6 down-regulated genes (with >5 fold-change and p < 0.005) upon 6 hours of stimulation. At 24 hours, the effects of PMA-ionomycin on genes such as BMP's, CSF's, IFNγ, IL1β, IL6, IL11, TGFβ and TNF are continuously observed, while the effect on other genes such as interleukin 2, 3, 5, 9, 10, 13, 17 and 22 diminishes twenty-four hours after stimulation (Figure 8 and Table 6). To validate these results, the protein levels of 8 selected cytokines secreted by the PBMC was measured using a multiplex ELISA array (Figure 9). The effects of these mRNA expression changes were observed in the changes in cytokine production induced by PMA ionomycin at 6 hours after stimulation. The induction in cytokine production by PMA-ionomycin was sustained up to 48 hours after stimulation, despite the observation of the subdued mRNA expression for some cytokines at 24 hours after stimulation.

Figure 8: RNA isolated from resting PBMC or PBMC stimulated with PMA ionomycin for 6 or 24 hours were characterized on the Human Common Cytokine PCR Array.

Log fold-changes in gene expression between PBMC stimulated with PMA ionomycin and resting PBMC are plotted against t-test p-values to produce a “volcano plot”. The higher the position, the more significant the gene's fold-change. Genes plotted farther from the central axis have larger changes in gene expression. Thresholds for fold-change (vertical lines, 5-fold) and significant difference (horizontal line, p < 0.005) were used in this display.

Using the Common Cytokine PCR Array, we identified 29 genes that exhibited at least a five-fold change in gene expression between resting and PMA ionomycin stimulated peripheral blood mononuclear cells at 6 hours after stimulation. Our data show that changes in cytokine mRNA levels detected by PCR Arrays accurately predict changes in protein levels measured by ELISA. Hence, the PCR Array offers a simple, reliable and sensitive tool for multiple cytokine profiling.

Figure 9. The effects of PMA-ionomycin on the secretion of the eight selected cytokines were assessed by multiplex cytokine ELISA. As shown in the above graphs, in parallel with the PCR Array results (upper panel), a marked increase in cytokine release (lower panel) was seen for IL-13, and IFN-g and TNF-α. The induction in cytokine secretion by PMA-ionomycin were sustained up to 48 hours of stimulation, despite the observation of the subdued mRNA expression for some cytokines such as IL-13 and TNF-α after 24 hours of stimulation.
Table 6. List of cytokines induced or down regulated in Phorbol Myristate Acetate Ionomycin-stimulated Peripheral Blood Mononuclear Cells (PBMC) versus resting PBMC.

The significance of the change in gene expression between the two samples was evaluated by unpaired Student t-test for each gene. The level of statistical significance is set at <0.005. Genes that show at least a five-fold difference in expression between the two samples are listed in the table. After six hours of stimulation, a total of 29 genes have at least a 5-fold change in expression between the stimulated and resting PBMC, with 23 genes having increased expression and six genes having decreased expression in stimulated PBMC.

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Summary:

The RT² Profiler PCR Array System is the ideal tool for analyzing the expression of a focused panel of genes. The flexibility, simplicity, and convenience of standard SYBR Green PCR detection methodology make the PCR Array System accessible for routine use in any research laboratory. The correct combination of instrument-specific plate format and master mix matches the PCR Array System with the most popular real-time instrument platforms. The arrays feature a pathway-focused or a customizable gene content design, while demonstrating the sensitivity, specificity, and reproducibility performance expected of real-time PCR. The focused design of this system decreases the amount of time necessary to complete the experiment and facilitates easier and more straightforward data analysis. Using this system, results can be generated with as little as 25 ng or as much as 5 μg of total RNA starting material. The specificity of the system guarantees the amplification of only one gene-specific product in each reaction meaning that the expression level result confidently reflects only the gene of interest. The reproducibility of the system (with intra-lab and inter-lab correlations greater than 0.99 and 0.97, respectively) demonstrates that the same results are obtainable by multiple end-users. As a result, the RT² Profiler PCR Array System is indeed ideally suited to allow every laboratory to combine the performance of real-time PCR with the profiling capabilities of a microarray.

References:

PCR Array Buyer’s Guide

Step 1: Find your pathway in the list below. For complete PCR Array gene lists, see our web site at: www.SABiosciences.com/ArrayList.php

Step 2: Determine which PCR Array format fits the instrument in your lab using the Real-Time PCR Systems table.

Step 3: Select your pack sizes and reagents. Place your order by phone, fax, or e-mail:
Phone: 888.503.3187 Fax: 888.465.9859 E-mail: order@SABiosciences.com

<table>
<thead>
<tr>
<th>Pathway / Topic Focus</th>
<th>PCR Array Catalog Number</th>
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<tbody>
<tr>
<td>Angiogenesis</td>
<td>PAXX-024Y</td>
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<tr>
<td>Angiogenic Growth Factors &amp; Angiogenesis Inhibitors</td>
<td>PAXX-072Y</td>
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<tr>
<td>Apoptosis</td>
<td>PAXX-012Y</td>
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<td>Atherosclerosis</td>
<td>PAXX-038Y</td>
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<tr>
<td>Breast Cancer and Estrogen Receptor Signaling</td>
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<td>cAMP and Calcium Signaling Pathway</td>
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<tr>
<td>Cancer Drug Resistance and Metabolism</td>
<td>PAXX-004Y</td>
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<td>Cancer PathwayFinder™</td>
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<td>Cell Cycle</td>
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<td>MAP Kinase Signaling Pathway</td>
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<td>Custom Options</td>
<td>Inquire</td>
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“XX” = HS, MM, RN (Human, Mouse, Rat) see web site for availability

Real-Time PCR Systems

Determine the plate type and master mix that fits your real-time PCR system.

<table>
<thead>
<tr>
<th>Instrument Make and Model</th>
<th>PCR Array Plate Format (Cat. No. Y = )</th>
<th>Required Master Mix</th>
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<td>ABI 7500 FAST Block</td>
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<td>ABI 7700 (Perkin Elmer)</td>
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RT² SYBR Green qPCR Master Mixes are required for use with PCR Arrays.

Pack Sizes and Required Reagents
Volume discounts are built into the PCR Array 12-pack and 24-pack sizes.

<table>
<thead>
<tr>
<th>PCR Array Pack Sizes</th>
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<td>Two (2) 96-well PCR Arrays</td>
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<td>Twelve (12) 96-well PCR Arrays</td>
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<tr>
<td>Twenty-Four (24) 96-well PCR Arrays</td>
<td>PA-01#-24</td>
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</tbody>
</table>

RT² First Strand Kit (Cat. No. C-02, enough for 12 reactions)

RT² qPCR-Grade RNA Isolation Kit (Cat. No. PA-001, enough for 12 RNA isolations)