RT² Profiler PCR Arrays: Pathway-focused gene expression profiling with qRT-PCR

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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. The RT² Profiler PCR Array System combines the quantitative performance of SYBR® Green real-time PCR with the multiple-gene profiling capabilities of a microarray. The RT² Profiler PCR Array is a 96- or 384-well plate containing RT² qPCR Primer Assays for a set of 84 related genes, plus 5 housekeeping genes and 3 controls. The complete system includes an instrument-specific master mix and an optimized first strand synthesis kit. This paper presents experimental data showing that RT² Profiler 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 real-time PCR. This system brings together the quantitative performance of real-time PCR and the multiple-gene profiling capability of microarrays. Each RT² Profiler 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 RT² Profiler PCR Array System.

RT² qPCR Primer Assays are key components in the RT² Profiler 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 nonspecific products. The qPCR assays used in RT² Profiler PCR Arrays are optimized to work under standard conditions, enabling a large number of genes to be assayed simultaneously. Their specificity is guaranteed when RT² SYBR Green qPCR Mastermixes are used as part of the complete RT² Profiler PCR Array System protocol.

Contents

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 RT² Profiler PCR Array System.

Experimental protocol

Figure 1 depicts an overview of the RT² Profiler PCR Array procedure. Simply isolate RNA from your samples and convert to cDNA using the RT² First Strand Kit. Then aliquot this mixture (25 µl for 96-well or 10 µl for 384-well plates) to each well of the same RT² Profiler PCR Array plate containing the pre-dispensed gene-specific primer sets, and perform PCR. Use your instrument's software to calculate the threshold cycle (C_T) values for all the genes on each RT² Profiler PCR Array. Finally, calculate fold changes in gene expression for pairwise comparison using the $\Delta\Delta C_T$ method. A simple examination of C_T value consistency for the housekeeping genes quickly indicates the proper normalization method. A similarly rapid evaluation of the built-in RNA quality control elements provides the relative levels of genomic DNA contamination and inhibitors of either the reverse transcription or the PCR itself.

How it works

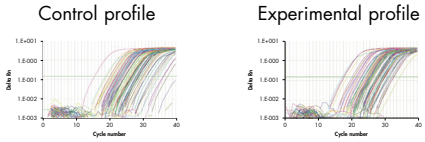
1. Isolate RNA from cells, tissues, FFPE and/or blood

2. Convert total RNA to cDNA

3. Add cDNA to RT² SYBR® Green Master Mix

Aliquot mixture across RT² Profiler PCR Array

4. Run in your real-time PCR instrument



5. Data analysis

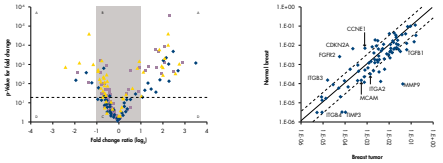


Figure 1. The complete RT² Profiler PCR Array procedure is easy to use and requires minimal hands-on time.

RT² Profiler PCR Array design and gene content

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 RT² Profiler 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 100 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.

	1	2	3	4	5	6	7	8	9	10	11	12
A	Gene 1	Gene 2	Gene 3	Gene 4	Gene 5	Gene 6	Gene 7	Gene 8	Gene 9	Gene 10	Gene 11	Gene 12
B	Gene 13	Gene 14	Gene 15	Gene 16	Gene 17	Gene 18	Gene 19	Gene 20	Gene 21	Gene 22	Gene 23	Gene 24
C	Gene 25	Gene 26	Gene 27	Gene 28	Gene 29	Gene 30	Gene 31	Gene 32	Gene 33	Gene 34	Gene 35	Gene 36
D	Gene 37	Gene 38	Gene 39	Gene 40	Gene 41	Gene 42	Gene 43	Gene 44	Gene 45	Gene 46	Gene 47	Gene 48
E	Gene 49	Gene 50	Gene 51	Gene 52	Gene 53	Gene 54	Gene 55	Gene 56	Gene 57	Gene 58	Gene 59	Gene 60
F	Gene 61	Gene 62	Gene 63	Gene 64	Gene 65	Gene 66	Gene 67	Gene 68	Gene 69	Gene 70	Gene 71	Gene 72
G	Gene 73	Gene 74	Gene 75	Gene 76	Gene 77	Gene 78	Gene 79	Gene 80	Gene 81	Gene 82	Gene 83	Gene 84
H	HK1	HK2	HK3	HK4	HK5	GDC	RTC	RTC	RTC	PPC	PPC	PPC

Figure 2. Layout of the cataloged RT² Profiler PCR Arrays. 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 RT² Profiler 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). These elements verify the efficiency of the RT reaction with a qPCR assay that specifically detects template synthesized from the RT² 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.

Why RT² Profiler PCR Arrays?

- **Simplicity:**
  The simplicity of RT² Profiler PCR Arrays makes expression profiling accessible for routine use in every research laboratory with a real-time PCR instrument.

- **Performance:**
  RT² Profiler PCR Arrays have the sensitive, reproducible, specific, and reliable performance to accurately profile multiple genes simultaneously in 96- or 384-well formats.

- **Relevance:**
  RT² Profiler PCR Arrays focus on profiling the genes relevant to the pathways or disease states of your interest.

Pathway-focused RT² Profiler PCR Arrays

The 96- or 384-well format of the RT² Profiler PCR Arrays is uniquely suited to our 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 array's comprehensive list of genes and to continually expand the breadth of available pathways, we utilize a systematic process comprising literature surveys, database searches, expert review, and user feedback. QIAGEN now has the largest collection of pathway- and application-specific human, mouse, and rat PCR arrays available on the market. (For examples, see Table 1.) This knowledge-based design merges the benefits of hypothesis-driven and discovery-based research, allowing researchers to answer highly specific questions in a systematic fashion. These pre-designed application-specific PCR arrays accelerate, simplify, and improve life science research by saving time, effort, and resources. Currently, RT² Profiler PCR Arrays are available for many pathways including apoptosis, inflammation, signal transduction, cancer, and other diseases. Visit the SABiosciences web site (www.SABiosciences.com/ArrayList.php) for a complete list.

Table 1. Examples of cataloged pathway-focused RT² Profiler PCR Arrays

Research application	RT² Profiler PCR Array	Gene content selection for the RT² Profiler PCR Array example
Biological process	Human Apoptosis	TNF ligands and their receptors BCL2 family members Caspases Death and effector domains ATM and p53 pathways
Functionally or structurally related genes	Mouse Common Cytokines	Interferons and interleukins Bone morphogenetic proteins Tumor necrosis factors Other various growth factors
Signal transduction pathways	Human NFκB Signaling Pathway	Extracellular ligands and receptors NFκB and IκB family members Kinases Transcription factors Responsive genes
Disease	Human Cancer PathwayFinder	Cell cycle control and DNA damage repair Apoptosis and cell senescence Cell adhesion Angiogenesis Invasion and tumor metastasis

Customized RT² Profiler PCR Arrays

For researchers who have special gene expression profiling needs, we offer a streamlined custom design and array production service. Custom RT² Profiler PCR Arrays provide researchers the flexibility to 1) verify a focused panel of genes identified by a high-density, genome-wide microarray, 2) modify the gene content of an existing RT² Profiler 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 RT² Profiler PCR Arrays. The content of an 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 RT² Profiler PCR Arrays are also available in either 96- or 384-well plate formats.

The complete RT² Profiler PCR Array System: Why the RT² Profiler PCR Array System works

The complete RT² Profiler PCR Array System includes the RT² Profiler PCR Arrays, the RT² SYBR Green qPCR Mastermixes 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 RT² Profiler PCR Array plate formats and the master mixes containing the appropriate reference dyes also provide RT² Profiler 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 RT² Profiler PCR Array that helps test the quality of the input RNA material.

Component 1: RT² qPCR Primer Assays

The greatest challenge for the RT² Profiler 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. We have 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 are:

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 ensure that the primers do not amplify bacterial genomic DNA, a common but minor contaminant of many *Taq* DNA polymerases.

2. Uniformity: So that the same annealing temperature may be employed for every well in each RT² Profiler PCR Array, only primer pairs with similar GC content, melting temperature (Tm), and other chemical and physical properties are used.

3. Efficiency: Short amplicons (~100–200 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 nonspecific annealing events.

Component 2: RT² qPCR Mastermixes

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 Mastermixes utilize a unique and proprietary chemically-modified HotStart *Taq* polymerase which only gains full activity after its heat activation step. Under these conditions, nonspecific priming events occurring at low temperatures are not extended. Other master mixes often amplify the resulting templates into nonspecific products which can cause false positive results. In addition, the RT² qPCR Mastermixes 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 Mastermix formulation is the foundation for the guaranteed specificity of the assays on the RT² Profiler PCR Array.

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 same simple two-step, 30-minute reaction. A proprietary genomic DNA elimination buffer completely removes any residual genomic DNA from your RNA sample. The buffer's optimized formulation also allows direct use of this RNA preparation for reverse transcription and finally real-time PCR without affecting reaction performance. Because genomic DNA contamination is eliminated, 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 RT² Profiler 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 Mastermixes and subsequent gene expression analysis with the RT² Profiler PCR Arrays as part of the complete RT² Profiler 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.

Table 2. Features of the complete RT² Profiler PCR Array system

Array design	84 pathway-focused genes
	5 housekeeping genes
	1 Genomic DNA control
	3 Reverse Transcription Controls (RTC)
	3 Positive PCR controls (PPC)
Primer design	Specificity: sequence alignment filter
	Uniformity: consistent melting and annealing temperatures
	Efficiency: short amplicon sequence
Master Mix	Instrument-specific SYBR Green formulations
	Supports all ABI, Bio-Rad, MJ Research, and Stratagene platforms
	Hot start enzyme: No extension of nonspecific priming events No amplification of secondary products like primer dimers
First strand synthesis	Optimized gDNA elimination buffer prevents false positive signals
	Built-in External RNA Control to test for inhibitors of RT

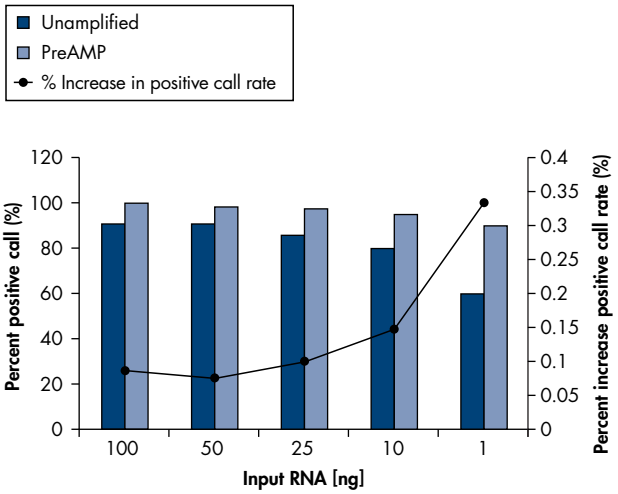


Figure 3. RT² Profiler PCR Arrays detect as little as 1 ng RNA. Different amounts of universal total RNA were characterized using the Human Inflammatory Cytokines and Receptors PCR Array (PAHS-011) with or without preamplification. The percentage of detectable genes was calculated for each RNA amount, with or without preamplification using the RT² PreAMP cDNA Synthesis Kit. The percentage of detectable genes was calculated for each RNA amount and the new pathway-focused PreAMP technology was found to enable detection with as little as 1 ng RNA.

RT² Profiler PCR Array performance:

Sensitivity

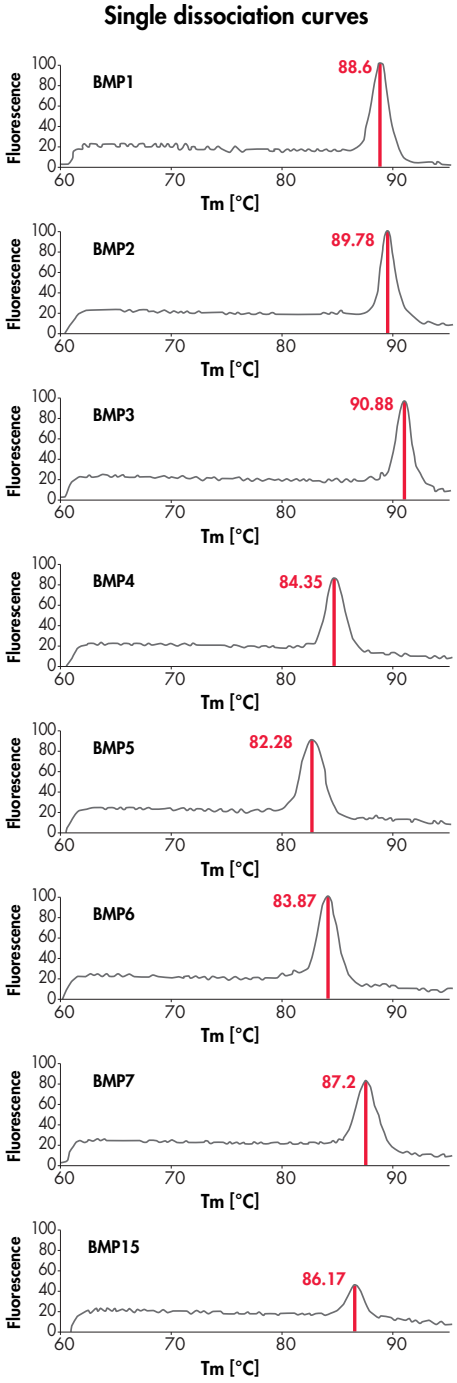
Researchers continually attempt to detect genes at ever lower levels of expression and in ever smaller amounts of total RNA. To meet these needs, the RT² Profiler PCR Array System must pass a very stringent test of sensitivity. A wide variety of universal RNA amounts were characterized with the RT² Profiler 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 C^T<35) versus the amount of input RNA. The results indicate that the RT² Profiler PCR Array System achieves greater than 80 percent positive calls with input total RNA amounts as low as 1 ng 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 1.0 µg to assure a maximum number of positive calls.

Specificity

The RT² Profiler PCR Array System has been designed and optimized for the SYBR Green detection method used by most real-time systems, making RT² Profiler PCR Arrays very flexible and widely applicable. Concerns have been raised over the specificity of SYBR Green detection and its ability to amplify only one gene-specific amplicon product, because it detects double-stranded DNA nonspecifically. Our experimentally verified primer design algorithm, used for the RT² Profiler PCR Arrays, guarantees the generation of single, gene-specific amplicons without the co-amplification of primer dimers or other nonspecific secondary products.

For an example of a stringent test of RT² Profiler PCR Array specificity, we characterized the real-time PCR dissociation curves of each gene on a RT² Profiler 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 RT² Profiler 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 RT² Profiler PCR Array System now brings a level of specificity to SYBR Green detection that most thought could be achieved only by more expensive probe-based methods.

A



B

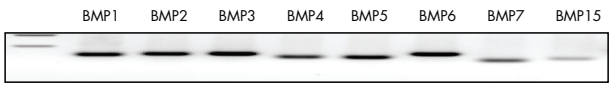
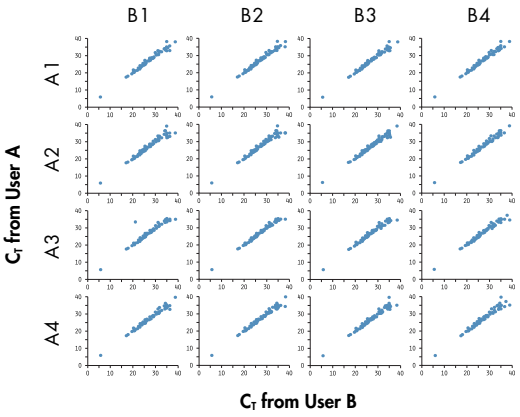


Figure 4. High specificity with the RT² Profiler PCR Array System. RT² Profiler PCR Arrays demonstrate a high degree of specificity for target genes. XpressRef Human Universal Total RNA (5 µg) was characterized on the Human TGFβ/BMP Signaling Pathway RT² Profiler PCR Array using the RT² SYBR Green/Fluorescein qPCR Mastermix 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).

Reproducibility

The quantitative nature of real-time PCR should impart a high degree of reproducibility onto the RT² Profiler PCR Array System. To test this reproducibility, 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 RT² Profiler PCR Array on two separate days. The raw threshold cycle values for the entire array's gene panel were then compared between each user's replicates and all four of the other user's replicates. Figure 5 displays the resulting scatter plots and correlation coefficients. Each comparison yields the predicted ideals of straight lines with slopes of 1.0 and correlation coefficients of 0.99 or greater. The results demonstrate the high degree of plate-to-plate, run-to-run, and replicate-to-replicate reproducibility inherent in the RT² Profiler PCR Arrays System technology, even at the level of raw data.

A



B

Correlation coefficients (R values)

	A1	A2	A3	A4
B1	0.993	0.989	0.995	0.992
B2	0.994	0.990	0.995	0.992
B3	0.992	0.990	0.993	0.992
B4	0.993	0.992	0.994	0.992

Figure 5. High user-to-user reproducibility. The RT² Profiler 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 RT² Profiler PCR Array and the RT² SYBR Green/Fluorescein qPCR Mastermix on the Bio-Rad iCycler. Panel A compares the raw threshold cycle values of the 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 B lists the correlation coefficient of the linear curve fit for each scatter plot comparison.

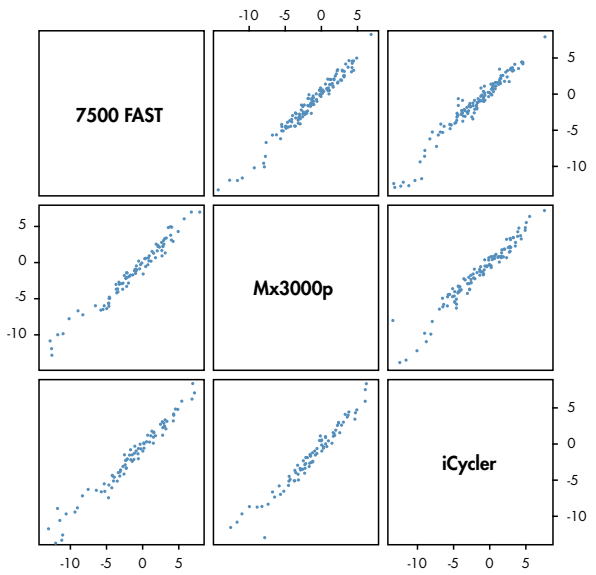
To directly demonstrate that the results from the RT² Profiler PCR Array System are indeed reproducible, the fold-differences in the expression of drug metabolism genes between two different RNA samples were compared across three different real-time PCR instrument platforms. In each gene expression profile 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 RT² Profiler 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.

Table 3. Typical performance of the RT² Profiler PCR Array

Sensitivity	80% positive call with as little as 25 ng
Dynamic range	At least five (5) orders of magnitude
Specificity	Primers amplify single, target-specific PCR products
Reproducibility	Correlation coefficients (R) ≥ 0.99 for intra-lab raw C_T values Correlation coefficients (R) ≥ 0.97 for inter-lab fold-change values Average standard deviation of 0.25 threshold cycles

A

Instrument-to-instrument reproducibility



B

Correlation coefficients (R values)

	7500 FAST	Mx3000p	iCycler
7500 FAST	1		
Mx3000p	0.980	1	
iCycler	0.981	0.973	1

Figure 6: High instrument-to-instrument reproducibility. RT² Profiler PCR Arrays demonstrate a high degree of instrument-to-instrument reproducibility. Two different MAQC RNA samples were characterized using the Human Drug Metabolism RT² Profiler PCR Array and either the RT² SYBR Green/Fluorescein qPCR Master Mix on the Bio-Rad iCycler 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).

RT² Profiler PCR Array application examples

Example 1: 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 RT² Profiler PCR Array and the RT² SYBR Green/Fluorescein qPCR Mastermix 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 RT² Profiler PCR Array and the RT² SYBR Green/Fluorescein qPCR Mastermix on the iCycler® PCR System.

Results: Gene expression profiling is important for discovering and verifying tumor biomarkers and therapeutic targets. Using the Cancer PathwayFinder RT² Profiler PCR Array and the Human Extracellular Matrix and Adhesion Molecules RT² Profiler 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 tumors were analyzed using the Cancer PathwayFinder RT² Profiler PCR Array. This 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 of the results from the Cancer PathwayFinder RT² Profiler 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. Upregulation was observed in 17 genes, while 7 genes appeared to be downregulated 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 downregulated, while the other five genes were upregulated. 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 RT² Profiler PCR Array.

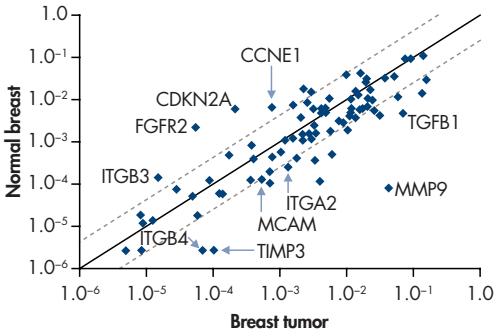


Figure 7. Relative expression comparison for 84 cancer-related genes between normal human breast and human breast tumor #1. The figure depicts a log transformation plot of the relative expression level of each gene ($2^{-\Delta Ct}$) between breast tumor (x-axis) and normal breast tissue (y-axis). The 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*

Gene	Fold change tumor/normal	t-Test p-value	Average raw C_T	
			Tumor	Normal
MMP9	542.45	0.0000	21.8	30.0
TIMP3	39.85	0.0000	30.5	35.0
TNF	35.51	0.0000	25.2	29.5
ITGA4	27.54	0.0001	31.1	35.0
TGFB1	15.10	0.0000	21.1	24.1
BCL2	12.27	0.0012	24.6	27.4
FOS	9.74	0.0003	20.1	22.5
GZMA	9.30	0.0003	25.5	27.9
TEK	6.88	0.0003	27.7	29.7
JUN	6.88	0.0008	22.3	24.2
APAF1	5.34	0.0018	23.8	25.4
ATM	5.34	0.0001	19.9	21.5
ITGA2	5.34	0.0042	26.8	28.4
PIK3R1	5.34	0.0001	21.3	22.9
SYK	4.65	0.0003	22.5	23.9
PLAUR	4.44	0.0007	26.4	27.7
MCAM	4.14	0.0000	28.2	29.4
PLAU	3.61	0.0132	27.8	28.8
ETS2	3.44	0.0015	23.5	24.4
ANGPT1	3.36	0.0028	31.3	32.2
FAS	3.36	0.0031	24.7	25.6
TERT	3.29	0.0314	34.1	35.0
NFKB1	3.07	0.0068	22.9	23.6
NME4	3.07	0.0019	24.1	24.9
ERBB2	-3.29	0.0000	25.9	23.3
ITGA3	-3.78	0.0000	23.9	21.1
UCC1	-4.65	0.0003	26.6	23.5
MYC	-5.34	0.0004	25.7	22.4
SNCG	-7.73	0.0000	26.0	22.2
CCNE1	-8.48	0.0000	27.6	23.7
ITGB3	-9.08	0.0026	33.3	29.3
CDKN2A	-26.91	0.0000	29.4	23.8
FGFR2	-41.74	0.0007	31.5	25.2

* 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 RT² Profiler 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 RT² Profiler 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 upregulation and 11 genes showing downregulation.

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 RT² Profiler PCR Array and the Extracellular Matrix and Adhesion Molecules RT² Profiler 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 RT² Profiler PCR Array focusing on Human Tumor Metastasis is available and could be used to continue this study.

Table 5. Changes in relative expression for genes encoding extracellular matrix and adhesion molecules between normal human breast and human breast tumor #2*

Gene	Fold change tumor/normal	t-Test p-value	Average raw C_T	
			Tumor	Normal
CTNND2	229.39	0.0000	23.8	31.6
TIMP3	104.57	0.0000	28.4	35.0
SELE	43.46	0.0000	26.3	31.7
MMP1	36.97	0.0000	27.9	33.0
MMP3	34.50	0.0000	29.9	35.0
KAL1	31.45	0.0000	23.1	28.0
MMP13	21.73	0.0000	26.9	31.2
MMP10	16.47	0.0000	31.0	35.0
MMP16	16.09	0.0000	25.3	29.2
FN1	11.92	0.0512	29.9	33.4
CD44	11.92	0.0046	23.5	27.0
TNC	10.87	0.0000	22.9	26.2
MMP9	10.62	0.0001	27.1	30.4
SELP	9.46	0.0001	26.1	29.2
MMP11	7.51	0.0000	25.0	27.9
COL7A1	7.00	0.0057	30.9	33.7
CSPG2	6.39	0.0000	24.0	26.6
COL4A2	5.56	0.0009	23.9	26.3
TNA	5.43	0.0001	26.9	29.3
COL11A1	5.31	0.0017	30.7	33.0
THBS1	4.84	0.0185	24.1	26.3
SELL	4.21	0.0002	24.7	26.7
HAS1	3.93	0.0010	27.5	29.4
CTNND1	3.84	0.0007	30.4	32.2
ITGA4	3.34	0.0000	25.4	27.1
ITGA7	3.34	0.0003	27.6	29.3
THBS2	3.19	0.0058	26.1	27.7
SPP1	-3.08	0.0000	23.6	21.9
ITGB5	-3.31	0.0000	23.2	21.4
CTNNB1	-3.31	0.0003	21.2	19.4
ITGAV	-4.57	0.0072	26.5	24.2
CNTN1	-5.25	0.0001	28.8	26.3
MMP7	-5.37	0.0000	25.7	23.2
ITGB3	-7.25	0.0094	32.1	29.2
ADAMTS1	-9.35	0.0003	25.5	22.2
LAMA3	-10.26	0.0000	24.7	21.2
NCAM1	-23.02	0.0000	30.9	26.3
ITGB4	-30.38	0.0000	26.6	21.6

* 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 (C_T) values seen in the two samples are also listed for comparison.

Example II: Monitoring cytokine expression levels

Materials and methods: Peripheral blood mononuclear cells (PBMC) were treated with or without 50 ng/mL PMA + 1 µg/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 triplicates using the Human Common Cytokine RT² Profiler PCR Array with the RT² SYBR Green/ROX qPCR Mastermix 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 $\Delta\Delta C_T$ method in the PCR Array Data Analysis template.

To verify the results obtained from the RT² Profiler PCR Array, the protein levels of eight selected cytokines secreted by the PBMC (IL-2, 4, 5, 10, 12, 13, and IFN-γ and TNF-α) were 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 ELISArray 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 RT² Profiler PCR Array System offers a simple, reliable and sensitive tool for multiple cytokine profiling. Using the Human Common Cytokines RT² Profiler PCR Array, we have monitored the mRNA levels of 84 different cytokines in stimulated versus untreated human peripheral blood mononuclear cells (PBMC).

The gene expression results identify 23 upregulated and 6 downregulated genes (with >5 fold-change and p<0.005) upon 6 hours of stimulation. At 24 hours, the effects of PMA-ionomycin treatment 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 24 hours after stimulation (Figure 8 and Table 6). To verify these results, the protein levels of 8 selected cytokines secreted by the PBMC were 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 treatment at 6 hours after stimulation. The induction of cytokine production by PMA-ionomycin was sustained up to 48 hours after stimulation, despite the observation of subdued mRNA expression for some cytokines at 24 hours after stimulation.

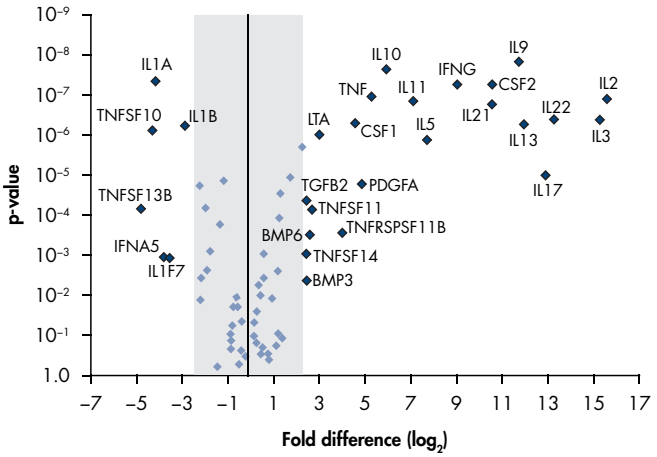


Figure 8. RNA isolated from resting PBMC or PBMC stimulated with PMA-ionomycin for 6 or 24 hours were characterized on the Human Common Cytokine RT² Profiler PCR Array. Log_2 fold-changes in gene expression between PBMC stimulated with PMA-ionomycin and resting PBMC are plotted against t-test p-values 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 Human Common Cytokines RT² Profiler 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 RT² Profiler PCR Arrays accurately predict changes in protein levels measured by ELISA. Hence, the RT² Profiler PCR Array offers a simple, reliable, and sensitive tool for multiple cytokine profiling.

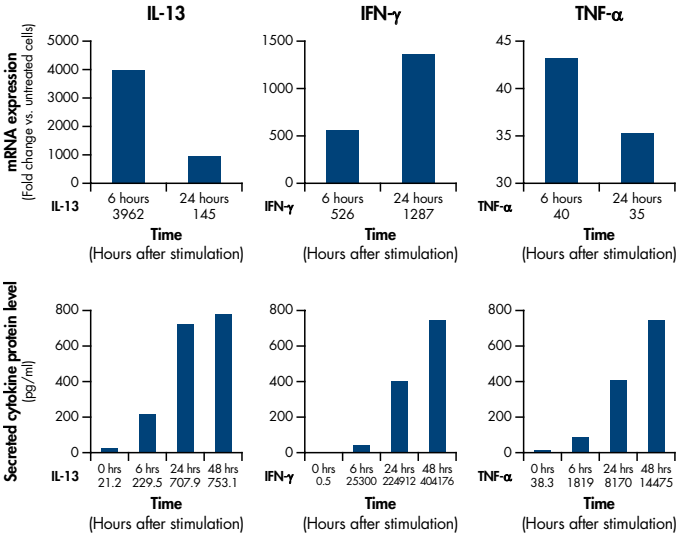


Figure 9. The effects of PMA-ionomycin on the secretion of the eight selected cytokines were assessed by multiplex cytokine ELISA. As shown in the above graphs, in parallel with the RT² Profiler PCR Array results (upper panel), a marked increase in cytokine release (lower panel) was seen for IL-13, IFN-γ, and TNF-α. The induction in cytokine secretion by PMA-ionomycin were sustained for 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 5. List of cytokines induced or downregulated in phorbol myristate acetate ionomycin-stimulated peripheral blood mononuclear cells (PBMC) versus resting PBMC *

	6 hours after stimulation				24 hours after stimulation			
	Average raw C_T value		Stimulated/Resting		Average raw C_T value		Stimulated/Resting	
Gene	Stimulated	Resting	Fold change	t-test p-value	Stimulated	Resting	Fold change	t-test p-value
IL2	14.64	29.99	47820.23	0.0000	13.54	26.91	11190.60	0.0000
IL3	19.53	34.56	38218.94	0.0000	18.46	30.35	4020.99	0.0000
IL22	21.08	34.14	9823.35	0.0000	24.26	30.62	87.02	0.0000
IL17	21.51	34.21	7601.14	0.0000	20.63	32.26	3365.64	0.0000
IL13	21.05	32.80	3961.96	0.0000	23.65	30.74	144.67	0.0000
IL9	23.49	35.00	3339.31	0.0000	22.22	31.15	516.75	0.0000
IL21	19.76	30.13	1522.26	0.0000	20.00	30.09	1152.06	0.0000
CSF2	16.80	27.15	1494.38	0.0000	15.53	26.86	2714.87	0.0000
IFNG	13.57	22.41	525.91	0.0000	13.94	24.19	1287.18	0.0000
IL5	21.89	29.40	208.71	0.0000	25.77	29.35	12.70	0.0000
IL11	24.22	31.12	136.74	0.0000	25.35	34.35	542.45	0.0000
IL10	21.43	27.21	62.77	0.0000	26.37	24.33	-3.87	0.0015
TNF	17.91	23.04	40.00	0.0000	18.69	23.72	34.54	0.0000
PDGFA	24.17	28.84	29.22	0.0000	23.27	28.05	29.11	0.0000
CSF1	21.27	25.64	23.73	0.0000	20.64	23.85	9.78	0.0000
TNFRSF11B	30.39	34.25	16.63	0.0003	30.63	32.16	3.06	0.0060
LTA	22.19	25.06	8.39	0.0000	20.26	24.76	23.92	0.0000
TNFSF11	26.61	29.10	6.40	0.0001	27.28	29.61	5.30	0.0001
BMP6	26.37	28.79	6.14	0.0003	26.40	29.28	7.84	0.0000
BMP3	31.45	33.71	5.50	0.0041	35.00	34.71	-1.16	0.1996
FASLG	20.90	23.16	5.46	0.0000	21.54	24.16	6.48	0.0000
TGFB2	28.98	31.23	5.43	0.0000	30.88	33.36	5.91	0.0029
TNFSF14	32.77	35.00	5.37	0.0009	33.51	35.00	2.98	0.0003
TNFSF8	20.16	22.27	4.92	0.0000	19.94	24.17	19.88	0.0000
TNFSF13	29.20	30.38	2.60	0.0000	31.80	26.02	-52.10	0.0000
BMP4	32.11	33.29	2.58	0.0935	28.99	32.54	12.38	0.0003
IL6	18.77	19.88	2.47	0.0002	19.92	22.49	6.29	0.0000
GDF10	33.11	34.08	2.23	0.1166	32.95	29.13	-13.30	0.0006
IL20	31.75	32.56	2.00	0.0117	32.27	35.00	7.03	0.0001
IL4	32.00	32.31	1.42	0.3010	33.36	32.22	-2.08	0.0025
TNFSF12	26.05	26.25	1.32	0.0057	29.28	23.84	-41.16	0.0000
IL12A	27.19	27.19	1.14	0.0971	27.18	27.18	1.06	0.3060
IL1F6	30.28	29.72	-1.29	0.2311	33.34	30.17	-8.48	0.0046
IL18	29.14	28.53	-1.33	0.0449	33.32	28.83	-21.26	0.0000
LTB	22.22	21.47	-1.48	0.0120	27.18	20.42	-102.54	0.0000
IL17C	28.78	27.95	-1.55	0.0213	31.86	27.66	-17.31	0.0001
IFNK	29.27	28.40	-1.60	0.0206	29.73	27.14	-5.71	0.0011
IL16	23.52	22.25	-2.11	0.0000	24.75	20.97	-12.91	0.0000
TNFSF4	28.43	26.89	-2.54	0.0002	27.96	25.45	-5.38	0.0000
IL1F9	29.69	28.07	-2.68	0.6977	26.92	22.81	-16.34	0.0000
IL15	29.46	27.55	-3.28	0.0007	28.79	26.32	-5.23	0.0000
IFNB1	31.11	29.07	-3.58	0.0022	34.37	30.03	-19.03	0.0015
BMP8B	29.36	27.25	-3.76	0.0001	31.35	28.51	-6.74	0.0018
IL12B	35.00	32.72	-4.25	0.0132	31.24	29.86	-2.46	0.0049
TGFA	29.29	26.92	-4.49	0.0000	27.96	24.06	-14.06	0.0000
IL1B	18.66	15.64	-7.12	0.0000	20.12	16.46	-11.93	0.0000
IL1F7	34.52	30.84	-11.19	0.0012	35.00	30.85	-16.76	0.0000
IFNA5	33.53	29.65	-12.89	0.0011	31.19	29.13	-3.93	0.0002
IL1A	24.27	20.02	-16.62	0.0000	25.48	23.24	-4.46	0.0000
TNFSF10	26.16	21.70	-19.22	0.0000	25.41	20.73	-24.20	0.0000
TNFSF13B	29.68	24.75	-26.62	0.0001	31.27	22.50	-411.10	0.0001

* The significance of the change in gene expression between the two samples was evaluated by unpaired Student's 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 show 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.

Summary:

The RT² Profiler PCR Array System is highly suited for analyzing the expression of a focused panel of genes. The flexibility, simplicity, and convenience of standard SYBR Green PCR detection methodology make the RT² Profiler PCR Array System accessible for routine use in any research laboratory. The correct combination of instrument-specific plate format and master mix matches the RT² Profiler PCR Array System with the most popular real-time instrument platforms. The arrays feature a pathway-focused or customizable gene content design, while demonstrating the sensitivity, specificity, and reproducibility expected of real-time PCR. The focused design of this system decreases the amount of time necessary to complete an 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 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.

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2. Perou, C.M. et al. (1999) Distinctive gene expression patterns in human mammary epithelial cells and breast cancers. Proc. Natl. Acad. Sci. USA. **96**, 9212.

The RT^2 Profiler PCR Array is intended for molecular biology applications. This product is not intended for the diagnosis, prevention, or treatment of a disease.

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