

# Detection of Low Abundance Transcripts on Nucleic Acid Microarrays Using Ultra-Sensitive Resonance Light Scattering (RLS) Particles

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October 16, 2002

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## Abstract

The advent of microarray techniques has enabled parallel analysis of up to hundreds of thousands of biological interactions. This has proved useful for many research areas, most notably in the area of gene expression analysis. Current limitations on the use of microarray analysis hinge on the sensitivity of the detection method used. The sensitivity of any microarray detection methodology is in large part limited by two key parameters. First, the quantity of lower abundance transcripts within the sample may be such that potentially, biologically relevant genes may be undetected. Second, the amount of starting material that is either available for use, or required by the detection method may be a limiting factor. As such, signal and/or target amplification approaches have been employed in attempts to address these limitations. These costly and time-consuming techniques can be problematic as they introduce the risk of bias to gene expression patterns. Ideally, detection methods for nucleic acid microarrays should allow for the measurement of low abundance transcripts in limited amounts of starting material, without the need for sample amplification. Resonance Light Scattering (RLS) Technology - a novel signal generation and detection platform, addresses these issues, thus improving the sensitivity of microarray analysis. Results indicate that when using RLS detection with 2  $\mu\text{g}$  input total RNA, a 15% - 40% increase in the number of positive features scored was observed when compared to fluorescence detection with 20  $\mu\text{g}$  input total RNA. Additionally, RLS detection was shown to be effective with as little as 0.1  $\mu\text{g}$  input total RNA sample. Gene expression microarray studies require sensitive, reproducible and easy-to-use labeling methods. Commonly utilized fluorescent labeling approaches lack the sensitivity required for analysis of rare transcripts without signal or target amplification, especially when starting sample material is limited (1,2,3). Target amplification can be used to increase the sensitivity of fluorescence detection, but this introduces a risk of bias. Due to these sensitivity limitations, relatively large amounts of total RNA, typically 10 - 20  $\mu\text{g}$ , are required to perform microarray experiments using a fluorescence approach without target amplification. The requirement for this level of input can be prohibitive for many investigators. Increasing the detection sensitivity with RLS Technology provides investigators with more gene

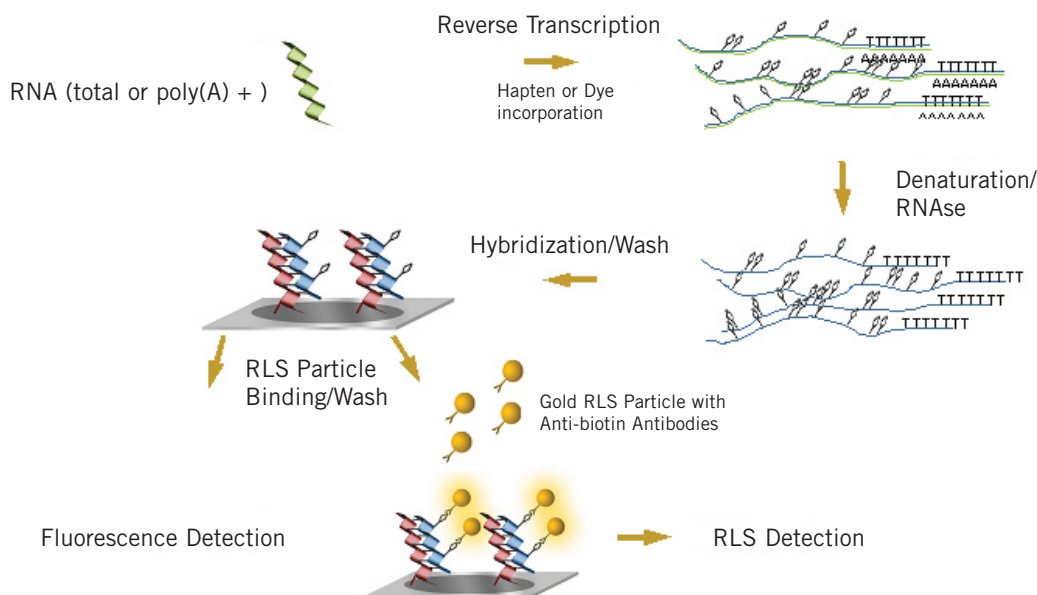
expression data, unbiased by sample amplification, while consuming less starting RNA sample. The reduced sample consumption enables the researcher to either carry out more experiments with a given amount of sample, or increase the number of experimental replicates, thereby increasing data reliability.

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## Introduction

RLS Technology, an ultra-sensitive, reproducible non-fluorescent signal generation and detection technology enables the detection of low abundance transcripts using less sample input material without amplification. RLS is based on nano-sized metal colloidal particles (RLS Particles) of uniform dimension that generate highly intense scattered light signals when illuminated with configured white light (4,5). RLS Particles can be used as ultra-sensitive labels for a wide variety of analytical bioassays. The colored light signal generated by a single RLS Particle is  $10^4$  -  $10^6$  times greater than the signal obtained from the most sensitive fluorescent molecules commonly used as labels in bioanalytical assays. The optical light scattering behavior of the particles is predictable based on proprietary algorithms that relate the signal intensity and color spectrum of the particles to their size, shape and composition. In addition, RLS signals are not subject to the effects of photo-bleaching or quenching, and as such are archiveable, forming a permanent record. To effect specific binding in analytical bioassays, the surface of RLS Particles can be derivatized with a variety of biomolecules, including proteins, antibodies, small molecule ligands, nucleic acids and oligonucleotide probes.

To demonstrate the sensitivity of RLS Technology when utilized for gene expression analysis on microarrays, an experiment was conducted with two objectives. The first objective was to investigate whether RLS detection could be used to score positive transcripts that would be below the threshold of fluorescent detection. The second was to determine whether the increased sensitivity of RLS detection could reduce the amounts of starting input material required, while still maintaining equal or superior results to fluorescence.



**Figure 1:** Illustration of detection of cDNA from reverse transcription of mRNA on nucleic acid detection microarrays via RLS Particle labeling or fluorescent labeling.

### Gene Expression Analysis on Microarrays

Gene expression was measured using labeled cDNA generated from various input levels (0.1 - 20  $\mu\text{g}$ ) of human placenta total RNA, hybridized to human 1.7K arrays. The arrays were generated by the University Health Network Microarray Centre and featured 1,728 human PCR amplicons arrayed in duplicate on Corning GAPS II slides. All experiments were done with 3 - 5 replicate arrays for each label type (biotin, Cy3 and Cy5) and input level. As arrays were only available in print lots of 70 slides, this study was split into two comparisons, one of RLS vs. Cy3, and another of RLS vs. Cy5 in order to maintain print lot specific comparisons between fluorescence and RLS. Anti-biotin 80 nm gold RLS Particles were introduced to the RLS microarray slides post-hybridization to bind to the biotin-labeled hybridized cDNA. The RLS signals on the microarray slides were collected as a TIFF image using the white-light/CCD based GSD-501 RLS Detection and Imaging Instrument. Fluorescent signals were collected as a TIFF image using the Axon GenePix 4000B laser scanner. Both the RLS and fluorescence experiments were performed in replicates of 10 arrays. Both RLS and fluorescence TIFF images were analyzed with ArrayVisionRLS image analysis software using Genicon Sciences' proprietary linear normalization feature.

### RLS Detection of Transcripts not Detected with Fluorescence

Previous microarray experiments using RLS detection indicated an approximate 10-fold reduction in input sample requirement as compared to fluorescence (data not shown)(6). Therefore, experimental results from the arrays described above were compared for RLS detection at an input level of 2  $\mu\text{g}$  total RNA and at an input level of 20  $\mu\text{g}$  total RNA for fluorescence detection. Positive features were scored as those with signals greater than 2.5 times the background (A.Thaliana negative controls), and are shown in Table 1 below.

RLS detection shows an increase in positive features compared to both fluorophores, even when using 10-fold less input total RNA. A 15% improvement (112 additional positives) was seen in the number of transcripts detected with RLS detection when compared to Cy3. The increased sensitivity of RLS detection was even more striking when compared to Cy5, where a 40% improvement was seen in the number of positives scored (184 additional positives).

RT-PCR was performed to confirm that transcripts scored as positive by RLS, but negative by fluorescent labels, had been

RLS vs. Cy3	Positive Features	
	#	% of total
RLS (2 $\mu\text{g}$ )	857	50%
Cy3 (20 $\mu\text{g}$ )	745	43%
<b>% Increase of positives (RLS/Cy3)</b>		<b>15%</b>

RLS vs. Cy5	Positive Features	
	#	% of total
RLS (2 $\mu\text{g}$ )	644	37%
Cy5 (20 $\mu\text{g}$ )	460	27%
<b>% Increase of positives (RLS/Cy5)</b>		<b>40%</b>

**Table 1.** Positive features detected by either RLS, Cy3 or Cy5 on 1.7K human PCR amplicon arrays using 2  $\mu\text{g}$  input total RNA for RLS and 20  $\mu\text{g}$  total RNA input for both Cy3 and Cy5.

accurately scored as positive by RLS. A randomly selected subset of 15 genes (fluorescence negative and RLS positive) were tested by manual quantitative RT-PCR to establish independent evidence demonstrating that mRNA from these genes was present in the starting sample. In addition, 10 co-positive genes (positive for both fluorescence at 20  $\mu$ g and RLS at 2  $\mu$ g detection), and 5 co-negative genes (negative for both fluorescence and RLS detection) were tested as controls. Additionally, both a -RT and a -template reaction were run as negative controls. Though the human placenta total RNA was treated with DNase I prior to purchase, a second DNase I treatment was performed as an additional precaution to remove any remaining genomic DNA contamination. The DNase I treated total RNA was then converted to cDNA using a poly d(T) primer and standard protocols. PCR reactions using gene specific primers and cDNA as a template were stopped every 5 cycles up to 40 cycles. The PCR products were separated on gels and quantitated by SYBR green staining. All 15 genes that were detected by RLS and scored negative by fluorescence produced measurable single PCR products of expected sizes between the 30th and 40th cycles. Table 2 summarizes these RT-PCR results from the various classes of genes tested.

Gene Category	Range of 1st PCR Cycle Detected	Number of Genes Tested
Fluorescence + / RLS +	20-35	10
Fluorescence - / RLS +	30	1
	35	12
	40	2
Fluorescence - / RLS -	Undetectable up to 40	5

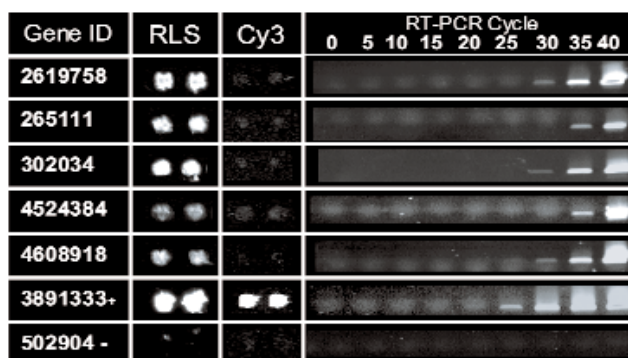
**Table 2:** Range of first detectable cycle by quantitative RT-PCR.

The RT-PCR results demonstrate that mRNA transcripts of the genes tested were present in the original RNA sample and that the RLS detection results were accurate in scoring these genes as expressed (i.e. true positive), whereas the fluorescence results incorrectly assigned those genes as not expressed (i.e. false negative). Moreover, as seen in Figure 2, the PCR products of the RLS positive genes were first detected at higher cycle numbers (30 - 40) by RT-PCR than the co-positive genes, thus suggesting that these targets were present at lower abundance levels, supporting the possibility that RLS detection can be used to identify lower abundance transcripts undetected by fluorescence.

### RLS Detection with Reduced Amounts of Input Material

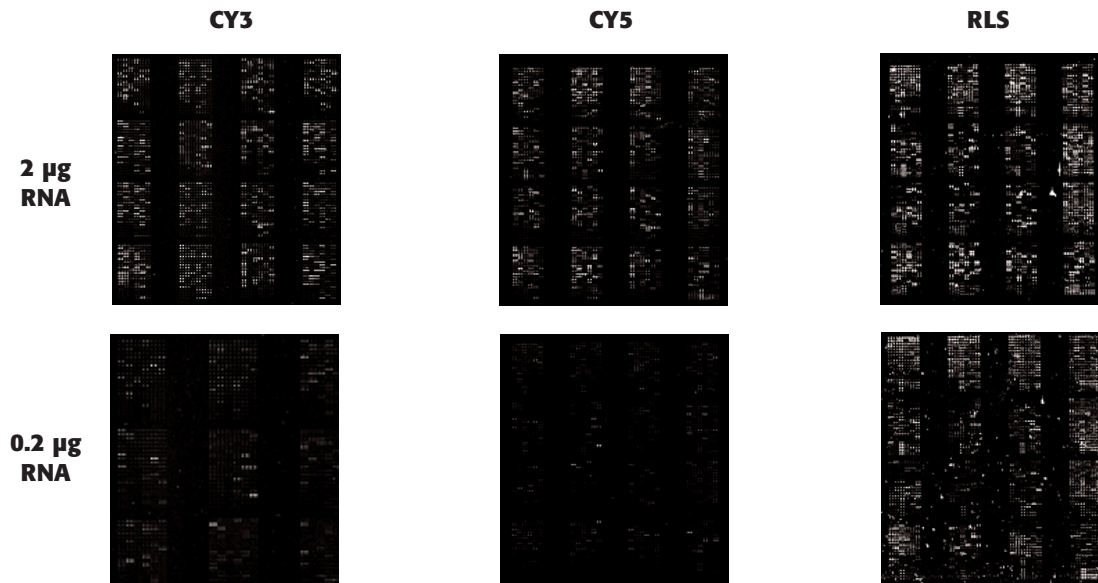
To investigate the performance of RLS detection in microarray experiments when using lower amounts of starting material, the experimental results from the titrated sample inputs were examined. The number of positive features scored using RLS detection was compared to the number of positives scored using fluorescence detection over an input range of 0.1 - 10  $\mu$ g total RNA. As described previously, to ensure that each comparison of RLS and fluorescence detection was performed within a single slide print lot, RLS was compared to Cy3 and Cy5 separately. Additionally, a single 0  $\mu$ g input level was processed as a negative control for RLS detection.

Representative images of microarrays at the 0.2  $\mu$ g and 2  $\mu$ g total RNA input levels are shown in Figure 3. Both RLS and fluorescence TIFF images were analyzed using ArrayVisionRLS



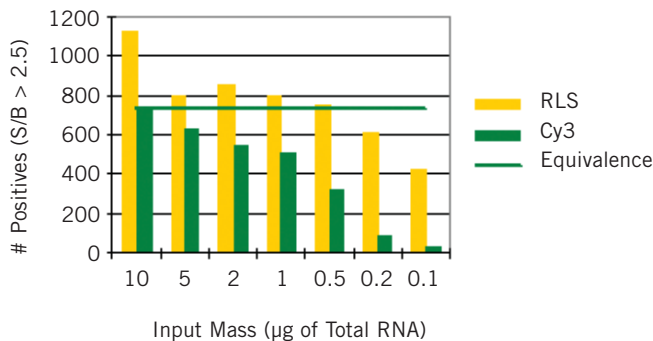
**Figure 2:** Representative RT-PCR results. 5 fluorescence - / RLS +, 1 fluorescence + / RLS +, and 1 fluorescence + / RLS + results are shown. TIFF images of the corresponding array features and RT-PCR products from the replicate reactions stopped at 5-cycle intervals are displayed.

image analysis software to examine the difference in signal to background across all the features on the microarrays. Figure 4 illustrates the number of positive genes obtained using different input masses of total RNA. Features were scored as positive when their signal intensities were measured to be 2.5 times greater than the mean gene intensity calculated for the 256 *A.thaliana* negative control features on the arrays. Even at the 10  $\mu$ g input mass of total RNA, RLS Technology enables the detection of a significantly higher number of transcripts when compared to fluorescence. This effect has greater significance as a lower input mass of total RNA is hybridized to the array. For example, less than 50% of the genes detected by the CyDyes at the 10  $\mu$ g total RNA input levels remain detectable when 0.5  $\mu$ g of total RNA is applied to the arrays. By comparison essentially all the genes detected by fluorescence at 10  $\mu$ g total RNA input level remain detectable by RLS at the 0.5  $\mu$ g total RNA input level. The equivalent input mass was defined as that amount of input total RNA that resulted in approximately the same number of genes detected as positive by RLS Technology as was detected by the maximum input of the respective fluorescent detection method. For detection by Cy3 and Cy5, the measured maximum number of positive features was estimated from the results obtained at the 10  $\mu$ g total RNA input levels. The RNA input level for RLS detection that was estimated to result in equivalent numbers of positive features was measured to be 0.5  $\mu$ g of total RNA. This determination is consistent with previous observations (data not shown) that the relative increase in detection sensitivity of RLS over Cy3 and Cy5 results in at least a 10-fold reduction in starting RNA requirements. Importantly, the expressed transcripts detected with Cy3 are always a subset of the larger number of transcripts detected by RLS. Furthermore, the transcripts detected by RLS detection at the lower levels of starting material are consistently detected at the higher input levels. This ability to use RLS with very small amounts of starting material would be particularly advantageous when sample is limited, such as when dealing with clinical samples or homogeneous cell populations obtained using laser capture microdissection or fluorescence activated cell sorting (FACS).

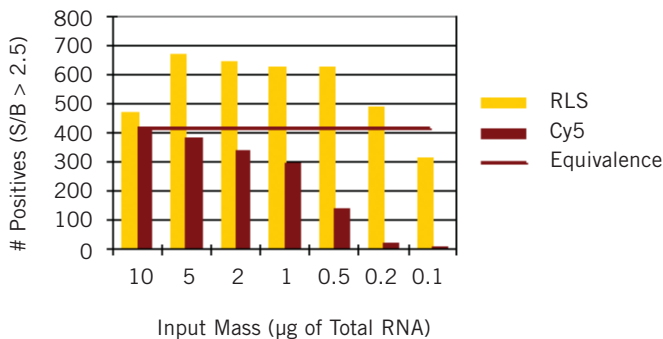


**Figure 3:** Image data of Cy3, Cy5 and RLS signal generation and detection on University Health Network, Oncology Gene Expression microarrays (screen stretch S/B 1 to 10).

#### # Positives vs. Input Mass - RLS vs. Cy3



#### # Positives vs. Input Mass - RLS vs. Cy5



**Figure 4:** Relative gene positivity rates for a range of input total RNA for RLS and fluorescence detection on University Health Network 1.7k human oncology microarrays.

## Conclusion

The results of this experiment confirm the increased sensitivity of RLS as compared to fluorescence detection when used for gene expression microarray analysis. Ultra-sensitive RLS detection enables new, RT-PCR confirmed positives to be detected that otherwise cannot be seen with either Cy3 or Cy5 even when using 10-fold less input total RNA with RLS detection. RT-PCR confirmation also indicated that these transcripts were representative of lower abundance genes. These lower abundance transcripts that are not detectable on microarrays with either Cy3 or Cy5, could potentially be biologically relevant genes that could be studied with RLS detection. Analysis of the sample input titration results identified that an even greater number of additional positive transcripts are detected with RLS. Furthermore, these sample input titration results showed that the number of positive transcripts detected at 10 µg total RNA with fluorescence detection can be achieved when using RLS detection at input levels of only 0.5 µg total RNA. Overall, RLS Technology is an ultra-sensitive signal generation and detection method that offers amplification-free analysis of low abundance transcripts and use of limited amounts of starting material for application in all gene expression microarray experiments.

## Materials and Methods

### Arrays

Two separate print lots of human cDNA microarrays containing 3,840 elements printed on Corning GAPS II slides (Corning Inc., Corning, NY) were purchased from the University Health Network Microarray Centre (Toronto, Canada). The 3,840 elements consist of 1,728 unique human cDNA clones spotted in duplicate, 256 elements derived from an *Arabidopsis thaliana* cDNA clone for use as negative controls and 128 elements of 3X SSC spotting buffer. The first print lot of microarrays used in the RLS vs. Cy3 comparison was post-processed by the

University Health Network Microarray Centre by UV crosslinking, washing in 0.1% SDS, and rinsing in DI water and isopropanol before drying. The second print lot was post-processed at Genicon Sciences by UV crosslinking only. The arrays were assayed using the One-Color Microarray Toolkit Protocol (Genicon Sciences, San Diego, CA).

### **cDNA Target Labeling and Purification**

#### **Reagents:**

The following reagents and materials were used to generate labeled target cDNAs: Total Human Placental RNA (Ambion, Austin, TX); Superscript II reverse transcriptase, RnaseOUT, 5X first-strand buffer, 0.1 M dithiothreitol, oligo d(T)<sub>(12-18)</sub> primers (Invitrogen, Carlsbad, CA); Cy3 and Cy5 dUTP (Amersham Pharmacia Biotech, Piscataway, NJ); Biotin-11-dUTP (Enzo Diagnostics, Farmingdale, NY); sodium hydroxide, Tris-HCl (Sigma, St. Louis, MO); QIAquick PCR Purification Kit (QIAGEN, Valencia, CA)

#### **Protocol:**

cDNA targets were prepared for each label type by direct incorporation using (per reaction), 7.5 µg total RNA, 0.75 µg oligo d(T)<sub>(12-18)</sub> and nuclease free water to 15 µl. The mix was incubated at 70°C for 10 minutes, chilled to 42°C for 2 minutes before adding reverse transcriptase mix (6 µl, 5X first-strand buffer; 3 µl, 0.1 M dithiothreitol; 0.9 µl RNaseOUT; 0.6 µl 50XdNTPs (25 mM dATP, DGTP, DCTP, 2.5 mM dTTP in the case of Cy3 and biotin-labeling reactions, 25 mM dATP, dGTP, dCTP, 10 mM dTTP in the case of Cy5 labeling reactions); 3 µl, 1mM Biotin-11-, Cy3 or Cy5 dUTP; 1.5 µl SuperScript II) and then incubated for 60 minutes at 42°C. The reaction was terminated by addition of 5 µl 1N NaOH and incubating for 10 minutes at 70°C, chilled and neutralized with 5 µl 1M Tris-HCl, pH 7.4. cDNA targets were purified using the QIAquick PCR Purification Kit using the recommended protocol and eluted with 50 µl buffer EB.

### **Pre-Hybridization, Hybridization, and Wash Conditions**

70 slides from each batch of microarrays were pre-hybridized according to the Genicon Sciences One-Color Microarray Toolkit's Pre-Hybridization Protocol (One-Color Microarray Toolkit, Genicon Sciences, San Diego, CA). From the first set of 70 slides, 36 were designated for Cy3 detection and 34 for RLS detection. From the second set of 70 slides, 36 were designated for Cy5 detection and 34 for RLS detection. The slides were rinsed in DI water and dried under a stream of clean, filtered air. Cy3 and Cy5 pools of labeled cDNA were divided into samples representing different input mass amounts (20 µg, 10 µg, 5 µg, 2 µg, 1 µg, 0.5 µg, 0.2 µg and 0.1 µg) of total RNA that were hybridized to the microarrays according to the Genicon Sciences One-Color Microarray Toolkit's Hybridization Protocol. Briefly, 3 slides at 20 µg total RNA input, 4 slides at 10 µg total RNA input, 4 slides at 5 µg total RNA input, 5 slides at 2 µg total RNA input, 5 slides at 1 µg total RNA input, 5 slides at 0.5 µg total RNA input, 5 slides at 0.2 µg total RNA input, and 5 slides at 0.1 µg total RNA input had their respective hybridization mixtures prepared, denatured at 95°C for 5 minutes, and applied utilizing LifterSlips (Erie Scientific, Portsmouth, NH). The pool of biotin-labeled cDNA was divided into samples representing different input mass amounts (10 µg, 5 µg, 2 µg, 1 µg, 0.5 µg, 0.2 µg, and 0.1 µg) of total RNA that were hybridized to the microarrays according to the Genicon Sciences One-Color Microarray Toolkit's Hybridization Protocol. Similarly, 4 slides

at 10 µg total RNA input, 4 slides at 5 µg total RNA input, 5 slides at 2 µg total RNA input, 5 slides at 1 µg total RNA input, 5 slides at 0.5 µg total RNA input, 5 slides at 0.2 µg total RNA input, 5 slides at 0.1 µg total RNA, and 1 slide at 0 µg total RNA for each of the slide batches had their respective hybridization mixtures prepared, denatured at 95°C for 5 minutes, and applied utilizing LifterSlips. All slides were incubated at 42°C for 16 hours in a hybridization chamber, and subsequently washed according to the Genicon Sciences One-Color Microarray Toolkit's Post-Hybridization Wash Protocol. The Cy3 and Cy5 labeled slides were rinsed in DI water and dried under a stream of clean, filtered air.

### **RLS Particle Binding**

RLS Particle Binding was performed according to the Genicon Sciences One-Color Microarray Toolkit Protocol (Genicon Sciences, San Diego, CA). Briefly, slides were blocked with RLS Blocking Solution and then 80 nm gold RLS Particles functionalized with anti-biotin antibodies were added for a 60 minute binding step. After a brief wash to remove non-specifically bound RLS Particles, a final rinse in DI water and drying under a stream of clean, filtered air, the slides were archived by dipping the slides into Archiving Solution.

### **Array Imaging**

Cy3 and Cy5 labeled arrays were read with a GenePix 4000B scanner (Axon, Union City, CA) at 10 µm resolution, 100% Laser Power, PMT voltage 700 and 600 volts (Cy3 and Cy5 respectively) to obtain maximal signal intensities at high target input without signal saturation. Total read time for each slide was approximately 1.75 minutes. RLS labeled arrays were read with a white light/CCD-based GSD-501 RLS Detection and Imaging Instrument (Genicon Sciences, San Diego, CA) at 10 µm resolution and 0.35 second exposure time. The total read time for each slide was approximately 0.5 minutes. The resulting images were analyzed using ArrayVisionRLS image analysis software (Genicon Sciences, San Diego, CA, and Imaging Research Inc., St. Catherines, Canada).

### **Analysis**

ArrayVisionRLS parameters used in the analysis include: MTM density as the principal measure with a MAD threshold of 6, spot segmentation enabled, obvious outliers confirmed visually, flagged, and excluded from analysis. Background was defined as the average MTM density from the 256 A. thaliana features on an individual slide (camera bias of 100 subtracted from all MTM density measures for RLS calculations). A signal to background (S/B) ratio greater than 2.5 was defined as a positively detected feature. The average number of detected features for a given input on a given batch of slides was calculated as an output for comparison between labels.

### **Quantitative RT-PCR**

Human placenta total RNA (Ambion, Austin, TX) was certified usable in RT-PCR as purchased. The samples of total RNA were treated a second time in-house with DNase I as an additional step to remove genomic DNA contamination in the samples by incubating 14 µg total RNA with 1.5 µl DNase I, 0.5 µl RNaseOUT and 4 µl 5X first-strand buffer for 15 minutes at 37°C. The reactions were heated to 95°C for 5 minutes to stop the reaction. An aliquot of 1.4 µg was removed for use as a reverse transcriptase negative control (-RT) while the rest of the total RNA was converted to cDNA by adding 1.5 µg

oligo d(T)<sub>(12-18)</sub> and water to 30  $\mu$ l. The reaction was heated to 70°C for 10 minutes, then cooled to 42°C before the addition of reverse transcriptase mix (5X first-strand buffer, 8.4  $\mu$ l; 0.1 M dithiothreitol, 6  $\mu$ l; 50XdNTPs {25 mM dATP, dGTP, dCTP, dTTP}; SuperScript II, 3  $\mu$ l) and incubated for 60 minutes at 42°C. The reaction was terminated by adding 10  $\mu$ l 1N NaOH and incubating for 10 minutes at 70°C, chilled and neutralized with 10  $\mu$ l 1M Tris-HCl, pH 7.4. Probes were purified using the QIAquick PCR Purification Kit using the recommended protocol and eluted with 50  $\mu$ l EB buffer. PCR reactions contained 125 - 250 nM each gene specific primer, 10 - 50 ng cDNA, 25  $\mu$ l 2X HotStarTaq Master Mix, up to 6 mM MgCl<sub>2</sub> and nuclease free water to 50  $\mu$ l. Replicate reactions were stopped every 5 cycles up to 40 cycles with a no template reaction and a -RT reaction run to 40 cycles serving as negative controls for each primer pair. The PCR products were separated on 10% TBE Novex gels, stained with SYBR Green I stain, imaged and quantified with an AlphaImager 2200 (Alpha Innotech Corporation, San Leandro, CA).

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