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Optimization of oligonucleotide microarray fabricated by spotting 65-mer

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Abstract

DNA microarrays currently provide measurements of sufficiently high quality to allow a wide variety of sound inferences about gene regulation and the coordination of cellular processes to be drawn. Nonetheless, a desire for greater precision in the measurements continues to drive the microarray research community to seek higher measurement quality through improvements in array fabrication and sample labeling and hybridization. We prepared oligonucleotide microarrays by printing 65-mer on aldehyde functional group derivatized slides as described in the previous study xxx. We could improve the reliability of data by removing enzymatic bias during probe labeling and hybridizing under a more stringent condition. This optimized method was used to profile gene expression patterns for nine different mouse tissues and organs, and MDS analysis of data showed both strong similarity between like samples and a clear, highly reproducible separation between different tissue samples. Three other microarrays were fabricated on commercial substrates and hybridized following the manufacturer's instructions. The data was then compared with in-house microarray data and RT-PCR data. The microarray printed on the custom aldehyde slide was superior to microarrays printed on commercially available substrate slides in terms of signal intensities, background and hybridization characteristics. The data from the custom substrate microarray generally showed good agreement in quantitative changes up to 100-fold changes of transcript abundance with RT-PCR data. However, more accurate comparisons will be come as more genomic sequence information is gathered in public data domain.

INTRODUCTION

High-throughput analysis of gene expression was made possible by the advent of microarray techniques. Over the past years, microarray analysis has been used in a broad range of areas such as target gene discovery [1], drug target validation and gene-drug relationship study [2, 3], profiling of tumors [4,5] and clarification of metabolic pathways [6,7]. Rather recently, gene expression profiling was exploited in the clinical field by predicting disease outcomes and deriving clinical parameters [8,9].

Simultaneous analyses of thousands of genes enable researchers to explore many new fields in biochemistry, genomics, cell biology and medicine. These large-scale studies are not practical with conventional verification methods of gene expression levels like reverse transcription-polymerase chain reaction (RT-PCR), *in situ* hybridization and Northern blot

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analyses. Only a modest number of genes can be analyzed by these methods. However, poor correlation between microarray-based measurements and measurements by conventional methods has been reported [10,11,12] and the level of accuracy of microarray data remains contentious. For many cases, it was observed that there were significant discrepancies in quantitative changes between microarray and RT-PCR data. Therefore, many issues related to the performance of various microarray formats and target labeling with regard to data quality and validation, remain to be resolved before microarray technology can become a robust analytical tool in general biological laboratories.

Recently, the MicroArray Quality Control (MAQC) project had been initiated in order to provide quality control of microarrays through standardization, and the first set of MAQC papers showed varying levels of inconsistency in the microarray data [13,14]. Here, we seek to characterize microarray performance and investigate the cause of discordance with RT-PCR data, using oligonucleotide microarrays fabricated as described in a previous study [15]. In this report, we demonstrate that the results obtained with these oligonucleotide microarrays, using labeling and hybridization methods optimized for this type of array are in good overall agreement with RT-PCR data that have <100-fold changes. Also, comparisons of performance of the same oligos printed on commercial substrates show that the custom slides and handling methods can provide more reliable data from gene expression experiments than is accessible using commercial substrates in the suggested fashion. Studies achieving the high levels of reproducibility and accuracy that can be attained with long oligonucleotides, as demonstrated in this study, would allow improvements in microarray data interpretation and allow inspection of biological processes involving more subtle alterations of transcript abundance than are currently practical.

MATERIALS AND METHODS

Preparations of Microarrays

Oligonucleotides (65-mer) representing 7500 mouse genes were printed on aldehyde group modified slides as described in the previous study [15] with a slight modification. After printing, Schiff bases were reduced by treating with sodium cyanoborohydride (NaCNBH_3 , 8 mg/mL in 0.05M PBS, pH 8.0) for 30 min. Microarray reading and image analysis were performed with DNA Microarray scanner (Agilent Technologies PN G2565BA) and DeArray software as described previously [16].

Commercial slides were purchased from Corning Inc. (UltraGAPS coated slides, Acton, MA), MWG Biotech Inc. (Pan Epoxy slides, High Point, NC) and TeleChem International Inc. (SuperAldehyde Slides, Sunnyvale, CA). These slides were printed in the same manner as in-house coated slides. The slides were then processed and hybridized according to the manufacturer's instructions. Atlas glass mouse 3.8 I microarray and Atlas PowerScript Fluorescent labeling kit were obtained from BD Biosciences Clontech (Palo Alto, CA). The microarray was fabricated using average 80-mer oligonucleotides for 3757 genes and was ready to hybridize.

Labeling and Hybridization Methods

Amino-allyl labeling method was adapted from the Brown lab web site (<http://cmgm.stanford.edu/pbrown/protocols/aadUTPCouplingProcedure.htm>). Total RNA (20 μg) was reverse transcribed with a poly d(T)12–18 primer. During cDNA synthesis, amino group-derivatized nucleotide was incorporated to the strand by using a 1.5: 3.5 mixture of 5-(3-Aminoallyl)-2'-deoxyuridine 5'-triphosphate (AA-dUTP) and dTTP. NaOH and EDTA were then added to the reaction to final concentrations of 0.2N and 0.05M respectively for hydrolyzing RNA. After the RNA hydrolysis reaction at 65°C for 30 min, the solution was

neutralized with 1N HCl. cDNA was purified by filtering four times through Microcon YM-50 (Millipore, Bedford, MA) with water and concentrated to 8 μ L. Sodium bicarbonate buffer (1 M) was freshly prepared and 1 μ L was added to the solution. N-hydroxysuccinimidyl ester of Cy3 or Cy5 (Amersham Pharmacia Biotech, Piscataway, NJ) was suspended in 12 μ L of DMSO and 1 μ L aliquot of this was added to the cDNA solution. After the dye coupling reaction for 1 hr, the labeled cDNA was purified with a MinElute reaction cleanup kit (Qiagen, Valencia, CA) according to the manufacturer's protocol. The Cy3 and Cy5 labeled cDNA were combined and mixed with 50x denhardt's blocking solution (1 μ L), mouse Cot-1 DNA (0.8 μ g), poly d (A) (9 μ g), yeast tRNA (4.5 μ g) and 2X hybridization buffer (Agilent Technologies PN G2558A) to a final volume of 45 μ L. Hybridization was set up with a 25 $\times$ 44 mm LifterSlip (Erie Scientific, Portsmouth, NH PN 25 $\times$ 44I-2-4775) that was sonicated for 2 min in H₂O and dried immediately before use. The microarray was then put into a hybridization chamber with 50 μ L of H₂O and hybridization was performed for 14–20 h in a 70°C waterbath having a built-in stirrer.

RNA extraction

Total RNA was isolated using TRIzol (Life Technologies, Rockville, MD) and Qiagen RNeasy Midi columns from mouse NIH3T3, JC cell lines, tissues and organs. After homogenizing samples in TRIzol, the mixtures were heated at 65 °C for 5 min and chloroform was then added. The protocol was essentially described by Jiang *et al.* [17]. Equal amounts of total RNA were pooled from 9 tissues and organs, and this was used as a reference RNA for mouse tissue gene profiling study.

Quantitative PCR

The validation of expression ratios was done by RT-PCR for 37 selected genes using the LightCycler instrument (Roche Diagnostics, Indianapolis, IN). RT-PCR was performed as described previously and PCR products were verified by electrophoresis in a 2% agarose gel.

Statistical Analysis

The multidimensional scaling (MDS) method was used by performing MDS in the MATLAB package to investigate the similarity between tissue samples. We also performed a hierarchical clustering on logarithmic values of gene expression ratios using an average-linkage clustering algorithm [18,19].

RESULTS AND DISCUSSION

Effects of extent of labeling on hybridization accuracy

In the previous study [15], we used a direct labeling method using Cy3- and Cy5-dUTP. Even though we found that this labeling method produced results on spotted oligonucleotide microarrays comparable to those obtained comparable to results on cDNA microarrays, some spots evidenced an intensity bias for Cy3 labeled targets over Cy5 targets. We hypothesized this might be the result of differential enzymatic incorporation biases against the Cy dye substituted nucleotides during target synthesis.

To avoid this influence, we utilized an indirect amino-allyl labeling method in which the same substituted nucleotide, allyl-amino dUTP (AA-dUTP) is incorporated during cDNA synthesis of both the reference and sample target. Subsequently, amino group modified cDNA is conjugated with N-hydroxysuccinimidyl esters of Cy3 or Cy5. If there is any bias from this method, it will be from different characteristics of fluorescent dye molecules themselves, allowing exact normalization of these differences by linear scaling. The aminoallyl labeling method has been reported to show higher labeling efficiency and improved reproducibility

[20,21]. Fig. 1A shows the fluorescence signal plot of a homotypic hybridization ($r=0.99$). The average coefficient of variance (CV) of pairs from the two normalized channel intensities was observed to be 5 - 6% for all spots across the entire signal range. Also, a dye reversal experiment (Fig. 1B) was conducted, and an improved result ($r=0.94$) was obtained when compared with the previous study ($r=0.88$). This improvement is also partially dependent on increased stringency during the hybridizations, resulting in more accurate measurement of expression differences (see below).

As the extent of dye incorporation into the target influences both the sensitivity of the measurement and the tendency of the target to produce noise, the effects of this variable were investigated. The dye incorporation rate was controlled by changing the ratio of AA-dUTP to dTTP during cDNA synthesis. As shown in Fig. 2, signal intensities after hybridization increased as AA-dUTP varied from 10% to 40%. In general, compressed expression ratios were observed with lower signal intensities. To investigate the cause for this phenomenon, a comparison of 20 genes which showed very similar calibrated ratios ($SD < 0.6$) over AA-dUTP ratio changes with another 20 genes exhibiting compression of expression ratios at lower extents of labeling ($SD > 6$). Irrespective of Cy3 or Cy5 dyes, fluorescence intensities from two channels were grouped into stronger and weaker intensities for each gene and normalized. Fig. 2A shows that the pair member with stronger intensity increases at the same rate as the pair member with weaker intensity for the genes showing similar calibrated ratios over different dye incorporation levels. On the other hand, for the genes showing compressed ratios, the pair member with the weaker signal was over 3 times higher than the pair member with the stronger signal with probes having 10% AA-dUTP (Fig. 2B). In this case, the raw intensities of weaker hybridization signals showed less than 1000 a.f.u. (arbitrary fluorescence unit) all over the AA-dUTP ratio changes indicating these were from mRNA of a low copy number (Fig. 2C). If a sample has low levels of transcript, the data extraction program tends to overestimate the expression level [22]. As shown in Table 1, RT-PCR experiments proved that microarray system showed overestimation of the less abundantly expressed transcripts, causing compressed ratios in the measurement of highly differentially expressed genes. This disparity in intensity was intensified when the hybridization signal intensity was lower, making high levels of substitution favorable. This favorability is limited by the tendency of excessive dye incorporation to cause detector saturation with the most abundant transcripts, resulting in the underestimation of the abundance of these mRNAs. When the probes derived from 20 μ g of total RNA using 30% and 40% AA-dUTP were used for hybridizations, 1202 and 1113 genes respectively showed over 2-fold different expression between two cell lines. When a probe prepared from 10 μ g of total input RNA and 40% AA-dUTP was used, 1096 genes showed over 2-fold differences. Although all the probes used for other experiments in this study were prepared by using 30% AA-dUTP and 20 μ g of total RNA, above results suggest that a smaller amount of total RNA can be used for hybridizations with further optimization of labeling.

Effects of temperature on hybridization accuracy

Most gene expression profiling studies using microarrays utilize hybridization temperatures of 42°C or 65°C. In general, 42°C is used with formamide based hybridization buffer and 65°C with aqueous/SSC buffers. In our previous study, we used 65°C for hybridization, and measured ratios from oligonucleotide microarrays showed a significant underestimation of gene expressions when compared with cDNA microarrays and RT-PCR data. To resolve these discrepancies, we compared hybridizations at 5 different stringency conditions by increasing temperature. Signal intensities were compared from these hybridizations as shown in Fig. 3. The results were normalized so that total signal intensities were the same for the 5 hybridizations tested at different temperatures. We choose 30 genes showing more than 5-fold differences at 70°C, and the Cy3 or Cy5 signal for each gene was then divided by the highest signal among the five Cy3 or Cy5 hybridization intensities. The relative stronger and weaker

signals for each gene were presented in Fig. 3A–D. Regardless of Cy3 or Cy5, fluorescence intensities between two channels were divided into stronger and weaker signals. Fig. 3D shows average intensities for 30 genes. For every spots, stronger intensities became stronger and weaker intensities were weaker over temperature increase. In general, the difference between stronger and weaker signals was greatest at 70°C, resulting in a larger fold difference as presented as relative calibrated ratios. The calibrated ratios showing less than 1 were inverted and the relative ratios were then calculated for presentation convenience. Stringent hybridization is thought to inhibit non-specific binding and makes more oligonucleotides accessible to specific binding. However, specific binding was reduced above 70 °C, while non-specific binding was increased in a small degree, resulting in compressed calibrated ratios. In a report by Drobyshev *et al.* [23], they also showed that non-specific binding was involved with cDNA microarray hybridization by means of controlling washing stringencies.

In addition, it was observed that a hybridization volume under LifterSlip had a great impact on calibrated ratios. When the LifterSlip was partially filled (ca. 60%), the most accurate measurement of microarray system was observed as compared with RT-PCR data. Fulfilling the space under the cover slip caused a high amount of ratio compression. Adey *et al.* [24] indicated that microarray sensitivity could be improved by mixing a very small volume of hybridization solution. Although our system does not have a mixing device for hybridization, it is thought that a small scale of local movement of hybridization solution will occur by a built-in stirrer of the waterbath and this will be facilitated when the space is partially filled.

Comparison with commercial slides

Three different coated slides were purchased and printed with the mouse oligonucleotides in our laboratory. Post-printing processes and hybridizations were performed following the manufacturers' instructions. Atlas glass mouse 3.8 I microarray (BD Biosciences Clontech) was ready for hybridization, and cDNA synthesis and labeling were performed by using Atlas PowerScript Fluorescent labeling kit. For all other slides, fluorescent cDNA preparation was done as described in Materials and Methods section and hybridizations were conducted with a JC-Cy3 and NIH3T3-Cy5 probe mixture. Graphs of scattered plots from normalized Cy3 and Cy5 fluorescence intensities are shown in Fig. 4. Among all microarrays, Atlas slide showed the lowest hybridization intensities in overall and sporadic dot-like background was observed (Fig. 5). We also hybridized the Atlas slide with the probe prepared in the same way as for other slides, and a similar result having lower signal intensities was obtained. As for Corning slide (Fig. 4B), background intensity was observed to be very low (300 a.f.u.) and it showed a good correlation with our slide for highly expressed genes. However, because it generally showed lower signal intensities than our slide (about 1/10), lowly expressed transcripts were not measured accurately and underestimation of expression differences was observed (Table 1). With TeleChem slide (Fig. 4C), 1,100–1,200 a.f.u. background was observed and spots having low signal intensities were not scored with good quality. Only 2881 spots had a maximum quality out of 7567 spots in comparison with 5686 highest quality spots from our slide. Fig. 4D shows the fluorescence intensity plot of a single hybridization conducted with MWG slide. Over 7000 spots showed signal intensities of above 500 a.f.u. from both channels and 7279 spots showed a maximum quality. However, a large portion of this fluorescence was from non-specific binding. A background noise with irregular morphology was observed almost all over the array area and it produced fluorescence from 10000 to 60000 a.f.u.. When compared with RT-PCR data (Table 1), MWG slide showed the highest degree of compression among all slides, failing to measure significant expression changes in many cases.

Quantitative PCR

Except for highly differentially expressed genes (over 100-fold), RT-PCR data showed a good relationship with microarray data (Table 1). As we described above, the level of gene

expression at both high and low ends are distorted due to fluorescence detector saturation and the normalization method of data extraction software. One of the two channel intensities would be very low or high for those highly differentially expressed genes, and calibrated ratios will therefore not be accurately measured from microarray experiments. For *Clast1* and *Gas5* genes, the ratios were shown compressed with RT-PCR experiment unlike the rest of genes and we further investigated these genes using public information domains. We obtained mRNA sequences for these genes from UniGene and LocusLink and examined the sequences in relative to oligonucleotides and RT-PCR primers. The oligonucleotide manufacturer's website reports that there are 12 different mRNAs for *Clast1*, but 6 forms are available from the public domain (AB031386, AF115426, AK007408, BC019539, BC022166, and NM_023056). Among these, AF115426 did not have a site for the antisense primer used in RT-PCR experiment. Another primer set for *Clast1* was chosen to present all the 6 mRNAs and used in RT-PCR. The new result showed that JC and NIH3T3 cell lines have over 400-fold different level of *Clast1* mRNA in comparison with ca. 10-fold difference with the first primer set. Whether primer sets represent all the splice variants makes the discordance between microarray and RT-PCR data and it may explain other cases. For *Gas5*, mRNA sequences available in the public database are X59728, AA230325, BC004622 and NM_013525, even though the Compugen website indicates that there are only 3 mRNAs. None of these has a site for the full length of oligonucleotide. X59728 and NM_013525 showed a partial match with 29 bases out of 65-mer and the other two do not have an oligonucleotide site. Except for AA230325, all other three mRNAs have sites for primers used in RT-PCR experiment. As genome sequences are more updated and more splice variants are entered into the public information sector, confirmatory comparisons can be made possible in the future. At present, every microarray data we compared showed significant transcript level changes if any, and concordance with RT-PCR data in the direction of transcript expression changes.

Tissue gene expression patterns

To verify the efficiency of this oligonucleotide microarray system for studying expression profiles, total RNA was extracted from 9 mouse tissues and organs, and sample probe was prepared using 30% AA-dUTP and Cy3 dye. Reference RNA was made by pooling equal amount of each RNA from 9 samples and labeled with Cy5. Both channel probes derived from 20 µg of total RNA were mixed and hybridized at 70°C overnight. Duplicate or triplicate experiments were performed for each tissue and organ. For all 20 hybridizations, 6034 spots out of 7566 showed the highest measurement quality and these data were used for MDS and hierarchical clustering analyses. Both MDS and hierarchical clustering revealed distinctive separation of each tissue and organ (Fig. 6). The reproducibility of the microarray results was also demonstrated. Therefore, this microarray system will significantly improve the reliability of gene expression profiling data in the future.

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Fig. 1.

(A) Typical scatter plot of the normalized Cy3 and Cy5 fluorescence intensity from JC: JC hybridization (single experiment). Plotted are 7302 spots which have highest measurement quality. (B) Average $\log_2$ (ratio) from JC: 3T3 hybridizations versus average $\log_2$ (inverted ratio) from 3T3: JC hybridizations. Each experiment was done in duplicate and a total of 3942 spots were plotted with highest quality in all four hybridizations. All hybridizations were done at 70°C.

Fig. 2.

Fluorescence intensity changes over dye incorporation rate during probe labeling. All hybridizations were done at 70°C. The normalization of fluorescence was performed for each gene in four hybridizations. The empty symbols represent weaker signal intensities and the filled ones for stronger intensities. The relative calibrated ratios were calculated and are indicated as circles. (A) Average of values for 20 genes which showed similar calibrated ratios ($SD < 0.6$) (B) Average of values for 20 genes which showed different calibrated ratios ($SD > 6$) (C) Average of Log_{10} (raw signal intensity) for each 20 genes.

Fig. 3.

Stronger (filled square) and weaker (empty square) signal intensity changes over temperature for highly differentially expressed genes between JC and NIH3T3. Hybridizations were performed at 5 different temperatures. The normalization of fluorescence was done so that all 5 hybridizations had the same total intensities. Signal intensities were then grouped into stronger and weaker fluorescence for each gene, and relative fluorescence signals were calculated. The relative calibrated ratios were also indicated over temperature (circle). (A) Dlk1 (B) Pem (C) Oasl2 (D) Average fluorescence intensities for 30 genes

Fig. 4.

In-house microarray was compared with three other microarrays prepared using commercial slides. Hybridization was done with a mixture of JC-Cy3 and NIH3T3-Cy5 labelings and each graph was from single hybridization. The genes with highest quality were plotted from total spots of 7566. (A) In-house microarray (n=5686) (B) UltraGAPS slide from Corning (n=4798) (C) SuperAldehyde from Telechem (n=2881) (D) Pan Epoxy from MWG (n=7279).

Fig. 5. JC-Cy3: NIH3T3-Cy5 hybridization on Atlas glass microarray. Out of 3888 spots, 102 showed highest quality.

Fig. 6.
 (A) MDS three-dimensional plot based on the overall gene expression in 9 different mouse tissues and organs (B) Hierarchical clustering dendrogram.

Table 1
Comparison of calibrated ratios obtained from RT-PCR and microarray experiments.

Gene	RT-PCR	NHGRI	Corning	Telechem	MWG
Psmb8	>10000	0.022 ± 0.002	0.033 ± 0.010	0.024 ± 0.003	0.22 ± 0.03
Mmp3	>10000	0.0030 ± 0.0023	0.016 ± 0.009	0.033 ± 0.016	0.26 ± 0.21
Crabp2	0.00029 ± 0.00005	0.0085 ± 0.0006	0.021 ± 0.006	0.034 ± 0.030	0.58 ± 0.04
Oas2	0.00033 ± 0.00007	0.037 ± 0.003	0.057 ± 0.005	0.053 ± 0.004	0.41 ± 0.34
EndoA	0.00083 ± 0.00011	0.0086 ± 0.0007	0.022 ± 0.002	0.013 ± 0.001	0.098 ± 0.024
NSG1	0.0030 ± 0.0021	0.088 ± 0.031	0.22 ± 0.15	0.078 ± 0.011	0.67 ± 0.17
Ifit3	0.013 ± 0.002	0.028	0.061 ± 0.012	0.038 ± 0.008	0.078 ± 0.007
Stat1	0.048 ± 0.009	0.069 ± 0.004	1.14 ± 0.06	1.22 ± 0.18	1.19
Nes	0.057 ± 0.007	0.17 ± 0.02	0.15 ± 0.02	0.11	1.14 ± 0.01
NMI	0.065 ± 0.012	0.11	0.13	0.13 ± 0.01	0.58 ± 0.42
ISG15	0.069 ± 0.012	0.049 ± 0.011	0.037	0.035 ± 0.002	0.22 ± 0.07
Eno3	0.090 ± 0.042	0.14 ± 0.01	0.17	0.19	0.87 ± 0.08
Clast1	> 0.14	0.044 ± 0.004	0.033 ± 0.003	0.035 ± 0.003	0.88 ± 0.13
Cstb	0.43 ± 0.02	0.69 ± 0.02	0.84 ± 0.01	0.69 ± 0.05	0.84 ± 0.05
Kip2	0.45 ± 0.14	0.85 ± 0.02	0.75 ± 0.20	n.a.	0.86 ± 0.04
Pex5	1.05 ± 0.03	0.97 ± 0.04	1.50 ± 0.05	1.40 ± 0.10	1.09 ± 0.15
Osbp	1.43 ± 0.03	1.28	1.43 ± 0.21	1.15 ± 0.58	0.98 ± 0.02
Gas5	2.66 ± 0.05	28.10 ± 6.09	5.02 ± 3.57	9.89 ± 0.58	3.09 ± 1.92
H2-Ke6	3.33 ± 0.49	3.83 ± 0.58	2.25 ± 0.16	2.06 ± 0.40	0.89 ± 0.04
Mmp11	3.44 ± 1.70	5.85 ± 1.15	1.81 ± 0.57	2.50 ± 0.05	1.40 ± 0.18
Hoxa2	4.92 ± 1.56	6.47 ± 1.81	1.08	5.96 ± 4.2	0.98 ± 0.09
Pla2g7	7.27 ± 1.40	7.83 ± 4.16	6.83 ± 1.18	17.40 ± 4.09	1.68 ± 0.84
Fbln2	8.85 ± 1.30	19.26 ± 1.00	12.73 ± 0.74	8.16 ± 0.25	7.07 ± 3.62
Igfbp6	9.78 ± 0.65	5.33 ± 0.35	9.64 ± 0.87	8.40 ± 1.52	3.36 ± 2.32
Fabp5	9.83 ± 1.90	6.78 ± 0.30	8.10 ± 0.15	7.78 ± 1.67	4.17 ± 2.01
Fosb	10.53 ± 0.90	13.25 ± 1.31	1.08 ± 0.04	0.77 ± 0.15	0.98 ± 0.06
Ptgis	> 9.76	14.52 ± 2.87	5.99 ± 5.05	11.73 ± 0.30	1.59 ± 0.07
Ephx1	31.51 ± 5.79	15.71 ± 1.71	18.50 ± 1.30	15.85 ± 3.17	4.94 ± 5.00
Bgn	38.41 ± 14.23	41.49 ± 9.35	45.52 ± 2.79	22.72 ± 8.05	1.30 ± 0.64
MD3	71.25 ± 27.95	46.02 ± 0.20	41.59 ± 6.68	29.87 ± 15.18	5.59 ± 2.64

Gene	RT-PCR	NHGRI	Corning	Telechem	MWG
Rora	87.76 ± 3.74	18.91 ± 3.22	1.53 ± 0.40	5.00 ± 0.68	1.23 ± 0.01
Thbs	> 100	233.95 ± 125.49	14.51 ± 16.49	35.70 ± 15.81	3.13 ± 1.96
Col5a2	135.56 ± 79.15	93.94 ± 26.80	54.50 ± 9.46	49.78 ± 24.29	6.06 ± 0.42
Pem	213.38 ± 12.13	34.11 ± 15.39	13.94 ± 12.31	42.00 ± 2.03	2.46 ± 1.18
Nid1	464.22 ± 108.99	52.21 ± 3.75	36.18 ± 4.87	29.21 ± 15.51	0.91 ± 0.09
Ebf	609.14 ± 482.76	68.02 ± 33.58	6.30 ± 0.76	26.95 ± 8.32	3.53 ± 2.83
Dlk1	> 8900	69.05 ± 0.36	37.17 ± 3.00	12.43 ± 1.79	5.26 ± 1.79