AFFYMETRIX®

Technical Note

Globin Reduction Protocol: A Method for Processing Whole Blood RNA Samples for Improved Array Results

Utilization of whole blood total RNA samples on GeneChip® expression arrays has shown reduced detection sensitivity compared with using fractionated blood samples (e.g., peripheral blood mononuclear cells, or PBMC[1]). This Technical Note describes the development of a Globin Reduction Protocol (GRP) that can be used to specifically reduce the α1-, α2-, and β-globin mRNA population in whole blood total RNA samples prior to target labeling to address this sensitivity issue.

To understand how relatively abundant globin mRNA impacts GeneChip array data, a model system was established, in which varying amounts of *in vitro* globin mRNA were added to cell-line total RNA prior to preparation of cRNA targets. This model system was used to develop the Globin Reduction Protocol.

To test the efficiency of the GRP, whole blood total RNA samples obtained using the PAXgene™ Blood RNA Isolation System (QIAGEN GmbH, Germany) were treated with the GRP protocol. Increased Percent Present calls and reduced variability were obtained on GeneChip arrays after this treatment, compared with untreated control samples.

This research protocol should be useful for scientists analyzing whole blood and seeking greater assay sensitivity. Users are encouraged to test the protocol with their own samples and determine which blood processing method best meets their needs.

Introduction

Expression profiling with microarrays is becoming increasingly important in clinical research where blood represents the most widely used and easily obtained sample type. However, expression profiling of blood samples presents many challenges since it is composed of heterogeneous cell types, and the different methods for handling and processing blood may induce changes in expression profiles *ex vivo*.

In order to simplify the blood handling process and preserve expression patterns in whole blood, the PAXgene™ Blood RNA Isolation System was developed by QIAGEN and PreAnalytiX. These collection tubes contain an RNA stabilizing agent and provide improved RNA stability over time with minimum manual manipulation relative to standard blood collection tubes.

While its ease-of-use is seen as a benefit, it has been observed that the expression patterns detected on GeneChip® arrays from PAXgene samples are not as informative as desired. Two primary differences between data obtained with PAXgene system compared with PBMC are: a) reduced Percent Present calls (reduced assay sensitivity) and b) increased variability among different individuals.

It has been observed that cRNA targets, which are amplified and labeled from PAXgene total RNA samples, contain a characteristic dominant band of approximately 700 bases, which is thought to be amplified from the abundant globin mRNA in whole blood. To understand how relatively large amounts of globin mRNA impact GeneChip array data, a model system was established. In this model system, varying amounts of *in vitro* globin mRNA were added to cell-line total RNA prior to preparation of cRNA targets. Subsequent array hybridizations clearly demonstrated that increased globin mRNA in the sample resulted in decreased Percent Present calls, decreased call concordance, and increased Signal variation.

The Globin Reduction Protocol (GRP) method was then developed to reduce the amount of globin mRNA from PAXgene total RNA in order to improve the data quality on GeneChip arrays. Results described in this Technical Note show that this globin reduction technique was able to:

- Significantly reduce globin probe set Signal intensities. As a result, the Percent Present calls increased at least nine percent to 40.8-46.8 percent.
- Increase detection sensitivity. Out of all probe sets that were originally called as Absent in PAXgene samples but as Present in matching peripheral blood mononuclear cell (PBMC) samples, approximately 60 percent of these transcripts were rescued and detected on the arrays after GRP treatment.
- Improve data consistency. Coefficient of variation (CV) analysis across different individuals also demonstrated that GRP-processed samples resulted in reduced sample variability compared with untreated PAXgene controls.
- Show highly reproducible results within technical replicates.

The Globin Reduction Protocol requires approximately 60 minutes of additional sample preparation time prior to target labeling. However, due to limitations with the number of blood samples available in this study, users may consider conducting

additional research to test the robustness of the procedure before adopting it for routine expression analysis. The Globin Reduction Protocol is described in the Appendix of this Technical Note. Depending on individual research goals, users are encouraged to evaluate various options of blood processing techniques, including GRP, before determining the most suitable protocol.

Results

TARGET LABELING FROM TOTAL RNA ISOLATED FROM PAXGENE, RED BLOOD CELL (RBC) LYSIS PROCEDURE, AND PBMC

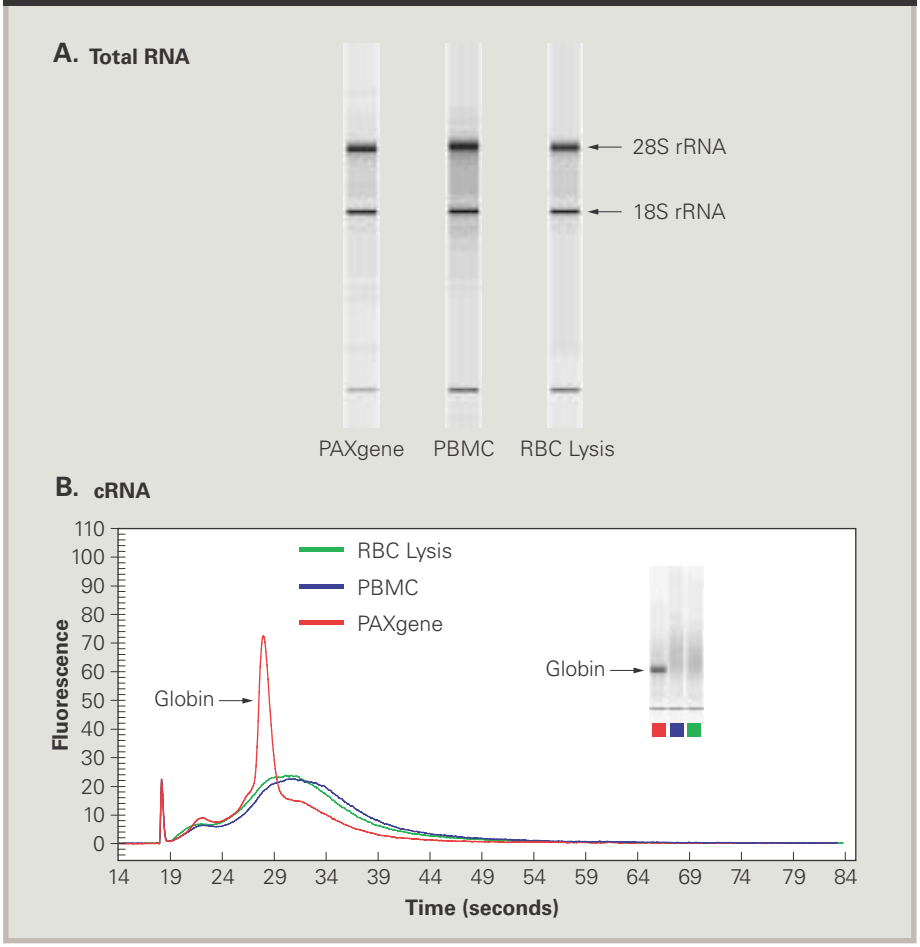
Total RNA was isolated from blood obtained from each individual after either Ficoll-Hypaque centrifugation (PBMC), RBC lysis, or with the PAXgene system. Figure 1A shows the Agilent 2100 bioanalyzer traces of the total RNA obtained from the three different preparations. The 28S and 18S rRNA peaks from all samples indicate a high degree of sample integrity. The data are representative of the five individuals shown later.

These total RNA samples were then used to prepare cRNA targets following the Standard Target Labeling Assay, as described in the *GeneChip® Expression Analysis Technical Manual* (available at www.affymetrix.com). The cRNA targets were analyzed on the 2100 bioanalyzer and the results are shown in Figure 1B. A significant difference was observed: the PAXgene sample contained a dominant peak at approximately 700 bases that was absent in the RBC lysis and PBMC samples.

The labeled cRNA targets were hybridized to GeneChip® Human Genome U133A (HG-U133A) Arrays. The Percent Present calls were greatly reduced in the PAXgene samples (average of 31.4 percent) relative to the PBMC and RBC lysis samples (47.4 and 45.4 percent on average, respectively).

The size of the dominant band in the cRNA traces and the very high Signal intensities on arrays suggested the strong cRNA band–only present in the PAXgene preparations–was amplified from globin mRNA. A model system was established to analyze, in more detail, the effect these abundant globin transcripts could have on subsequent array results.

Figure 1. Profiles of total RNA and cRNA obtained with three different blood RNA isolation methods. **A.** Total RNA was isolated by the PAXgene™ Blood RNA Isolation System (PAXgene), after red blood cell lysis (RBC Lysis), or after Ficoll gradient purification (PBMC). **B.** Labeled cRNA from the above three different blood RNA isolation methods. Gel representations of each individual bioanalyzer profile are shown in the inset. For both the total RNA and cRNA profiles, one-half microgram of total RNA or cRNA was analyzed on the Agilent 2100 bioanalyzer using the Agilent RNA 6000 Nano Assay (RNA 6000 Nano LabChip® Kit).

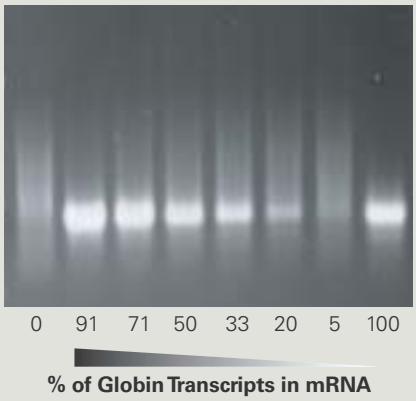


Globin mRNA was synthesized *in vitro* from cDNA clones and spiked into K562 erythroleukemia cell total RNA at varying ratios. In these samples, the globin mRNA constituted approximately 5, 20, 33, 50, 71, or 91 percent of the final mRNA pool. These spiked samples were then labeled for array hybridizations.

As seen in Figure 2, the exogenous globin cRNA became visible as a band on gel even at the second lowest level (20 percent). The samples containing 33, 50, and 71 percent globin mRNA produced profiles reminiscent of those targets generated from PAXgene-purified whole blood samples. Based on the relative intensity of the globin cRNA band on gel, it was estimated that, in a healthy individual, the globin mRNA may contribute up to 70 percent of mRNA from whole blood.

Array data are summarized in Figure 3, and clear trends are observed with increasing amounts of globin mRNA. The Percent Present calls (Figure 3A), Signal

Figure 2. Labeled cRNA from K562 cell total RNA spiked with varying amounts of exogenous *in vitro*-synthesized globin transcripts. Assuming that the mRNA content of total RNA in K562 cells was 2%, different amounts of *in vitro*-synthesized α1- and β-globin transcripts (at a 1:1 ratio) were added to make up a final globin composition to be between 0-91% of the total mRNA population. The RNA mixture was labeled (10 µg) and each labeled cRNA (2 µg) was run on a 1.2% agarose formaldehyde gel. The sample in the last lane on the right was prepared directly from the *in vitro* globin transcripts.



correlation with the control sample without globin mRNA spikes (Figure 3B), and call concordance relative to the control sample (Figure 3C) all decreased with increasing amounts of globin mRNA, while the False Change rate increased (Figure 3D). These results are consistent with the hypothesis that adding globin transcripts, constituting greater than 20 percent of the total mRNA population, severely impacts the array results.

GLOBIN REDUCTION PROTOCOL

The previous modeling experiments demonstrated the impact of large amounts of globin mRNA on GeneChip® microarray data. The results also showed that the effects could be substantially alleviated if the globin mRNA constituted less than 20 percent of the mRNA population in the sample.

The Globin Reduction Protocol was developed to specifically reduce the globin mRNA representation in the whole blood samples from up to 70 percent down to approximately 20 percent, in order to improve the sensitivity and to reduce variability observed on GeneChip arrays. A schematic diagram describing the principle of the protocol is illustrated in Figure 4.

In the GRP protocol, oligonucleotides are designed to anneal specifically to the target transcripts adjacent to the poly-A tails. Following hybridization of the RNA samples with the oligonucleotides, the samples are treated with RNase H, which selectively digests the RNA strand of the RNA:DNA hybrid, making these transcripts unavailable in the following oligo(dT)-primed reverse transcription reaction of the Target Labeling Assay.

In this study, specific oligonucleotides were designed for whole blood total RNA samples to target the highest expression globin transcripts, including α1, α2, and β globin. Several variables were examined for the assay, including oligonucleotide sequences and concentration, RNase H concentration, enzymatic digestion time, and temperature. Assay conditions were

Figure 3. Effects of increased exogenous globin transcripts on GeneChip® array performance in the K562 cell model system. Ten micrograms of labeled cRNA target were hybridized to Human Focus Arrays. **A.** Percent Present calls. **B.** Signal correlation of all probe sets, represented by linear correlation coefficient (R^2). Comparison analysis was carried out using the control sample (no spike-in globin mRNA) as the baseline, compared with samples containing varying amounts of exogenous globin transcripts. **C.** Call concordance of Present and Absent calls compared with control samples. **D.** False Change (%) compared with control samples where False Change was defined as an Increase call with a change in Signal Log Ratio ≥1 or a Decrease call with a change in Signal Log Ratio ≤-1.

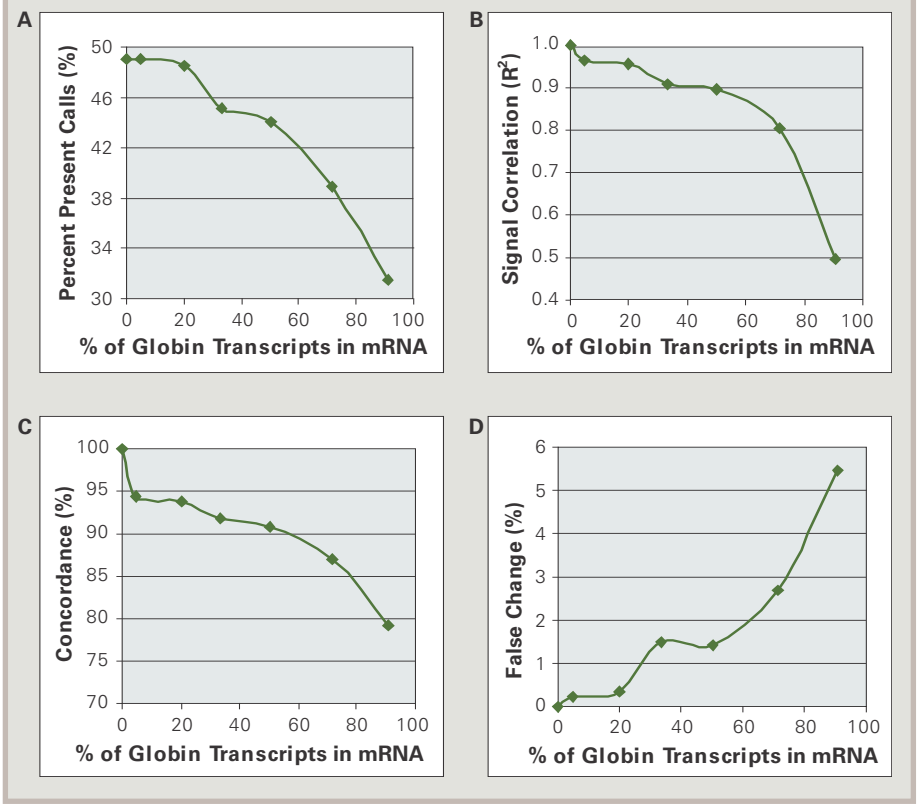
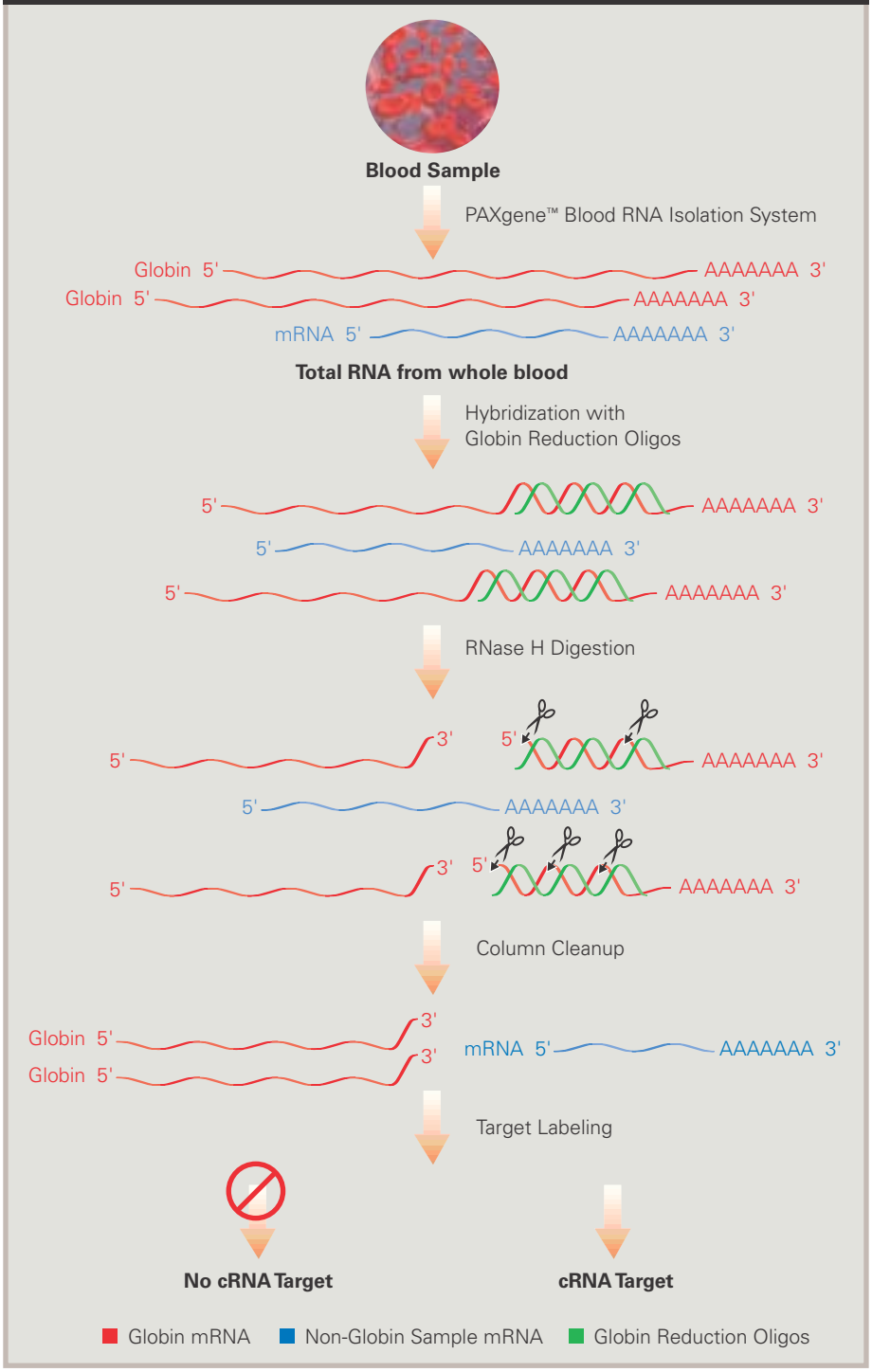


Figure 4. The Globin Reduction Protocol (GRP). Globin Reduction Oligos are added to whole blood total RNA and hybridized with globin mRNAs (α1, α2, and β). The oligos are complementary to the sequences adjacent to the poly-A tails of globin genes. The addition of RNase H specifically cleaves the region of the globin mRNA hybridized with the oligos, thereby dissociating the poly-A tail from the rest of the globin mRNA. The processed RNA is first column purified and then labeled following the GeneChip® Target Labeling Assay. A detailed procedure can be found in the Appendix.



optimized for a balance between α- and β-globin mRNA removal, specificity of the digestion, assay reproducibility, improved sensitivity, and reduced variability. Representative data are shown here and the detailed protocol is described in the Appendix.

GENECHIP® ARRAY DATA ON SAMPLES PROCESSED WITH THE GLOBIN REDUCTION PROTOCOL

To assess efficiency of the Globin Reduction Protocol (GRP), blood samples were drawn from five separate, healthy individuals. Total RNA was isolated from each blood sample by three methods: PAXgene, PAXgene total RNA treated with the Globin Reduction Protocol (PAX-GRP), and PBMC fractionation on Ficoll gradients. Blood samples from two of these individuals were also processed by an RBC lysis protocol, followed by total RNA isolation.

The total RNA samples were labeled, and the cRNA targets were analyzed by gel electrophoresis. As shown in Figure 5, the globin band, prominent in the PAXgene samples, was noticeably absent in the GRP-treated samples (PAX-GRP). The intensity of the cRNA smear derived from non-globin messages appeared to

Figure 5. Profiles of cRNA derived from GRP-processed PAXgene™ samples (PAX-GRP), PAXgene, and PBMC total RNA. For the three individual samples (1, 2, and 3) 0.5 µg each of the cRNA targets were analyzed by the Agilent 2100 bioanalyzer using the RNA 6000 Nano Assay (RNA 6000 Nano LabChip® Kit). Markers (RNA 6000 Ladder, Ambion) are shown on the left.

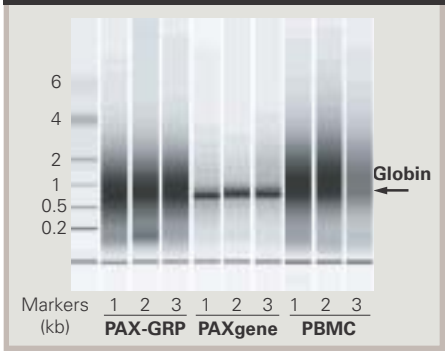


Table 1. Signal intensities for α- and β-globin probe sets before and after the Globin Reduction Protocol for five individual samples. Average Signal intensities (duplicate or triplicate sample preparations) for α1 globin (211699_x_at), α2 globin (211745_x_at), and β globin (211696_x_at) are shown from GeneChip® Human Genome U133A Arrays. All samples were purified by three protocols: PAXgene™, PAXgene followed by GRP (PAX-GRP) and Ficoll gradient centrifugation of PBMC (PBMC). Samples 1 and 2 were also purified by RBC lysis followed by TRIzol® purification (RBC Lysis).

		Purification Method			
	Sample	PAXgene	PAX-GRP	PBMC	RBC Lysis
Alpha 1 (α1) Globin	1	45,006	20,881	879	2,327
	2	22,407	16,862	531	2,862
	3	30,927	21,888	1,953	ND
	4	43,465	24,724	3,015	ND
	5	23,830	14,331	1,753	ND
Alpha 2 (α2) Globin	1	47,798	25,462	1,139	3,560
	2	22,332	18,646	562	3,928
	3	34,953	24,958	3,132	ND
	4	47,337	28,270	3,917	ND
	5	24,779	18,360	2,592	ND
Beta (β) Globin	1	36,976	21,453	1,214	3,054
	2	20,521	14,926	627	3,520
	3	27,333	14,564	3,287	ND
	4	34,431	18,397	3,642	ND
	5	19,629	8,427	2,233	ND

ND = Not done.

have been recovered by GRP treatment and was comparable to that of the PBMC.

The cRNA samples were then hybridized to GeneChip® Human Genome U133A (HG-U133A) Arrays. The Signal intensities for the globin probe sets were shown to decrease across all samples after GRP treatment, demonstrating the effectiveness of the procedure (Table 1). The median percent of reduction in Signal intensity due to the Globin Reduction Protocol was 39.9 percent for α1 globin, 25.9 percent for α2 globin, and 46.6 percent for β globin.

The sensitivity of the assay was then evaluated. In almost all cases with the PAXgene samples, the higher Signal intensity of globin probe sets correlated with lower Percent Present calls. As shown in Figure 6, the Percent Present calls increased 8.9-15.4 percent for the five individuals after the GRP treatment, and approached those obtained from PMBC or RBC lysis samples.

Protocol reproducibility was assessed by processing the same sample multiple times with the Globin Reduction Protocol. The array quality metrics were highly reproducible within the ranges expected without GRP. These metrics included Percent Present calls, Signal correlation ($R^2 \geq 0.95$), call concordance (>90%), and False Change (<0.1%).

One of the observations in this study was an increase in the GAPDH 3'/5' ratio after GRP treatment, which increased from approximately one to approximately two to three, in the five samples analyzed. This reduction in cRNA size may have been more sequence specific, associated to some degree with non-specific hybridization of GAPDH transcripts to the Globin Reduction Oligos.

To determine if the probe sets called as Present after GRP treatment were indeed detecting expressed genes and were not derived from experimental artifacts, the overlap between probe sets called as Present by the three different purification methods was calculated and illustrated in a

Figure 6. Percent Present calls for five individual samples. Ten micrograms of labeled cRNA target, generated from total RNA processed by GRP (PAX-GRP) or isolated by the different methods as described previously, were hybridized to GeneChip® Human Genome U133A Arrays. Average Percent Present calls generated by Affymetrix® Microarray Suite v5.0 software from technical replicates are shown.

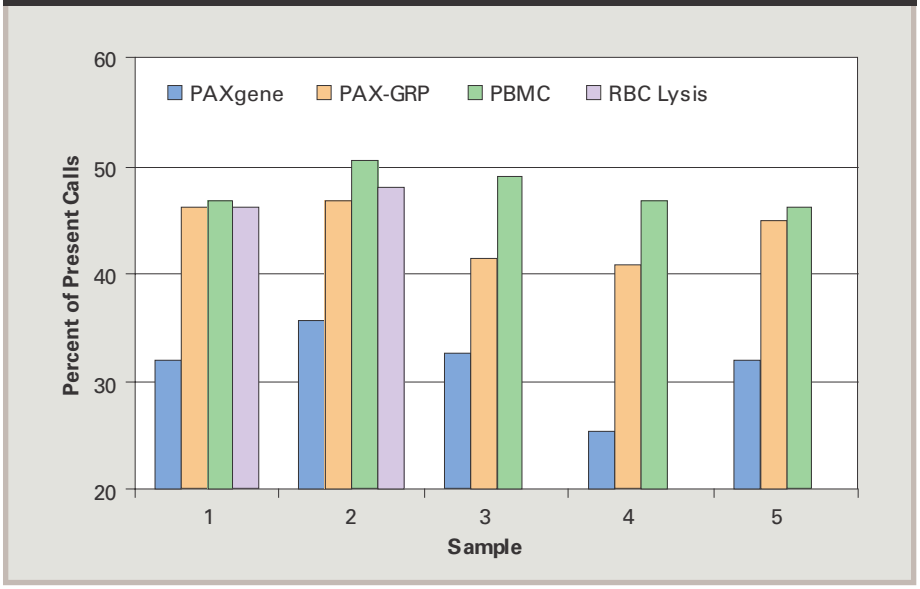
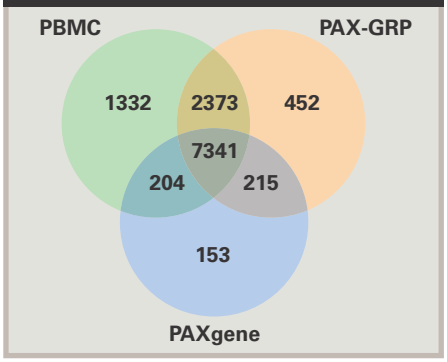


Figure 7. Venn Diagram illustrating the relationship of Present calls among the three purification techniques described previously.

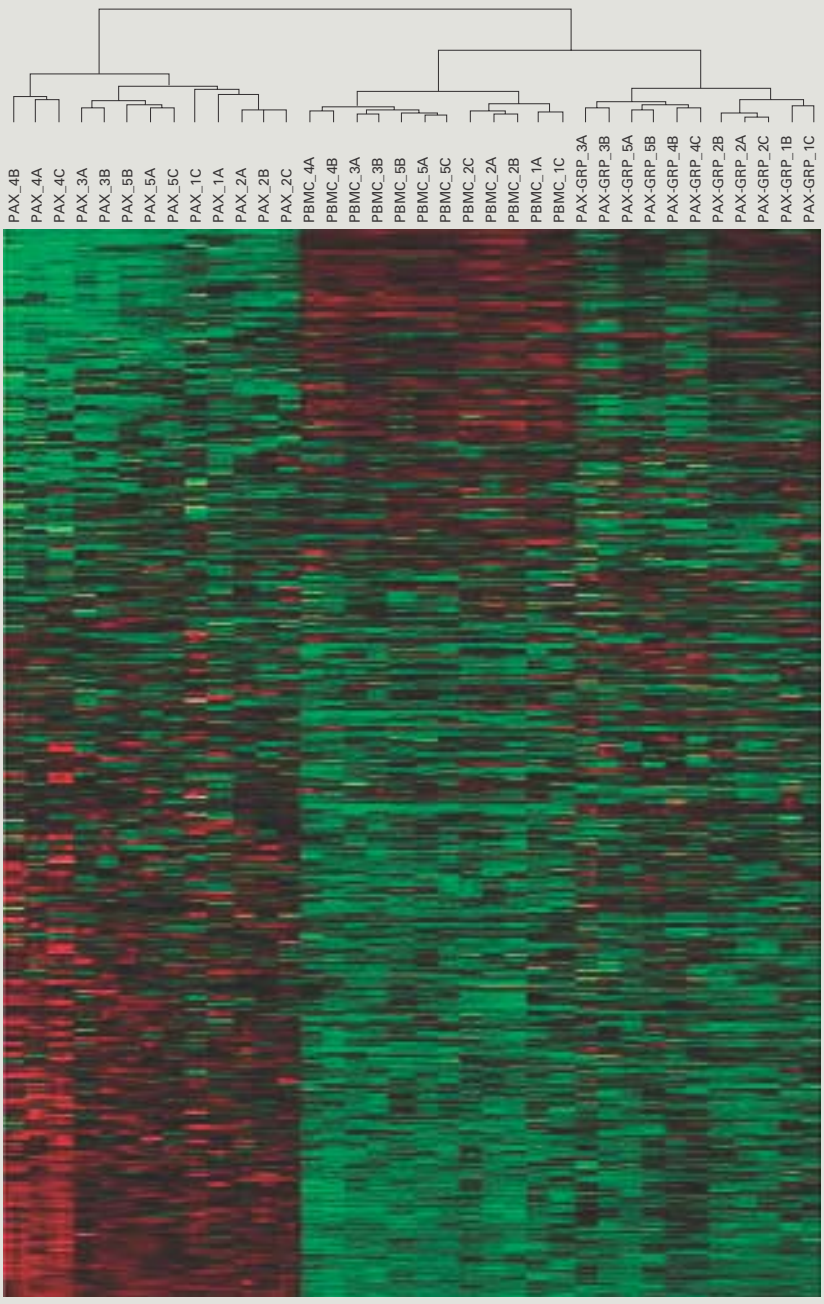


Venn diagram. As shown in Figure 7, the PAXgene purification followed by the GRP treatment resulted in 2,825 additional Present calls (2,373+452) relative to the original PAXgene sample. Of these recovered probe sets, 84 percent (2,373/2,825) were likely to be expressed since they were also called as Present in the PBMC fraction.

Approximately one-third of the probe sets that were called as Present in PBMC samples, but as Absent from PAXgene total RNA, continued to be called as Absent even after GRP treatment (1,332). Almost all of these probe sets had low Signal intensities and most likely represented genes expressed at a low level. While the Globin Reduction Protocol was effective for improving the detection of a large majority of genes expressed in the PBMC fraction, the final sample still represented a heterogeneous pool of different cell types. Residual mRNA species from red blood cells and other normal blood components——such as granulocytes—may have hindered the detection of these genes which were only detected in the PBMC fraction in this study.

Another way to show the relationship and global differences between the various purification techniques is to perform a hierarchical clustering analysis. Figure 8 shows such a diagram where the Signal intensities from all probe sets (approximately 22,000) were ordered for all of the

Figure 8. Heatmap, generated by GeneMaths (Applied Maths, Belgium), showing all probe sets on the GeneChip® Human Genome U133A Array as rows (approximately 22,000) and independent sample preparations as columns. Thirty-six experiments are shown in total including samples isolated from five individuals and processed with PAXgene, PAX-GRP, or PBMC. Technical replicates, in duplicates or triplicates, are represented by the same prefix followed by an A, B, or C. The ordering of the rows and the clustering of the columns was accomplished as follows: First, 50 was added to the Signal value and then the natural logarithm was taken. Second, each probe set was standardized to have zero mean and unit variance across the 36 arrays. Finally, the probe sets (rows) were ordered according to how well they met the pattern-matching criterion of being high in PBMC, high in PAX-GRP, and low in PAX. Therefore, those probe sets over-represented in PBMC and PAX-GRP, but under-represented in PAX samples, are concentrated on the top of the graph. Finally, hierarchical clustering was performed on the arrays (columns) using Euclidean distance and the Ward algorithm.



samples. As expected, a large set of genes showed higher relative expression (red) in the PBMC fraction than in the PAXgene samples. These probe sets were also intermediate in their expression in the PAX-GRP samples. Additionally, this set was expected to include those genes that were originally called as Absent in the PAXgene samples but were recovered after GRP treatment and called as Present, as represented in the previous Venn diagram.

Figure 8 also shows a large group of probe sets that were relatively highly expressed (red) in the PAXgene samples but were reduced (green) after the GRP treatment. These same probe sets also had lower expression (green) in the PBMC fraction. This is consistent with the notion that the removal of highly abundant globin transcripts affects the relative expression pattern of a large set of genes, as observed on GeneChip arrays.

Finally, the dendogram on the top of the graphic shows the relationship of the purification techniques. The PAXgene samples clustered together, as did the GRP-treated samples and the PBMC fraction.

Figure 9: PCA plot of the arrays, generated by GeneMaths (Applied Maths, Belgium). Green is PAX-GRP, red is PAX, and blue is PBMC. Shown are the projections of each array onto the top three principal components (PCs). 64.1% of the original variation is represented by the top three PCs (42.8% by PC1, 15.8% by PC2, and 5.5% by PC3).

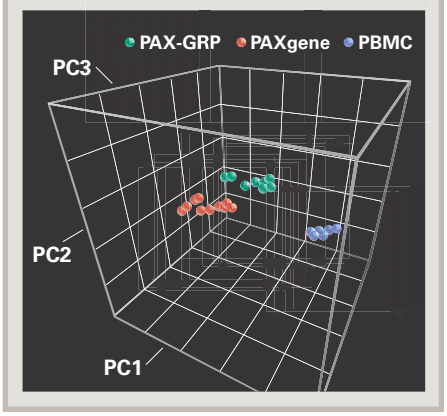
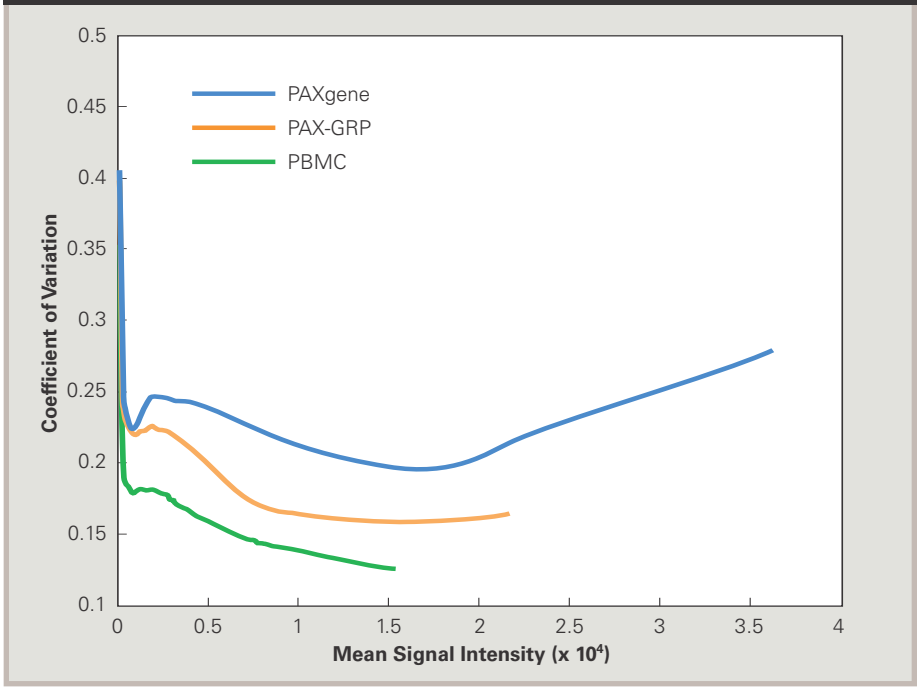


Figure 10. Coefficient of variation (CV) of Signal intensities for the three techniques (generated with MATLAB®, The MathWorks, Massachusetts, USA). CV is plotted as a function of mean Signal value for each of the three protocols: PAX-GRP, PAX, and PBMC. Each CV line was computed as follows: First, for each of the individuals, only one out of the two or three technical replicates was randomly selected to be included in the analysis. Second, for each probe set, the mean Signal value and the CV of the five individuals processed with the same technique were computed. This generated approximately 22,000 CV vs. mean Signal data points. Finally, loess smoothing was used to fit a curve to the data points.



A second method of analysis, Principal Component Analysis (PCA), shown in Figure 9, also supported the previous result. In this figure the blue spheres represent the PBMC samples, the red spheres the PAXgene samples, and the green spheres those samples purified by the PAXgene followed with GRP protocol. These results demonstrated that there are reproducible signatures produced by each of the purification techniques that outweigh the expression differences known to exist between individuals.

The three techniques were also compared for the assay variability among individuals. As shown in Figure 10, and also consistent with previous studies, the coefficient of variation (CV) of the PAXgene samples across the five different individual donors (blue line) was the greatest. Increasing CVs were also observed for the highest intensity probe sets. The PBMC samples (green line) exhibited the lowest CVs with the typical decrease in CVs at the higher Signal values. PAXgene samples treated with GRP (orange line) were intermediate in their CV levels demonstrating that the globin reduction resulted in reduced variability among individuals, compared with the PAXgene samples.

As a final check to determine if the expression patterns produced by the GRP protocol were an accurate view of expected expression patterns, a set of probe sets representing blood cell-specific signature genes was examined based on the relative distribution of Present calls as a function of purification procedure (see Venn Diagram, Figure 7). Markers called as

Present in the PBMC and PAX-GRP samples were attributed to lymphocytes and monocytes, as expected. Markers called as Present in the PAXgene and PAX-GRP samples were associated with granulocytes and red blood cells, which was consistent with the purification methodology. A few of the granulocyte markers were found only in the PAX-GRP samples. (The list of cell type markers is presented in a previous Technical Note, *An Analysis of Blood Processing Methods to Prepare Samples for GeneChip® Expression Profiling*.)

Discussion

While blood remains the most commonly used tissue in clinical research, its overall utility in expression profiling is sometimes hampered in that it contains a mixture of many cell types. In the case of PAXgene-isolated RNA from whole blood, the final sample appeared to be dominated by the red blood cell fraction, consisting of a high proportion of globin transcripts.

The Globin Reduction Protocol (GRP) described in this Technical Note was designed to improve the detection sensitivity for mRNAs from non-reticulocyte cell types. In a comparison of the PAXgene samples with or without treatment with GRP, GRP was shown to improve Detection call rates. Among the set of probe sets, originally called as Absent in PAXgene samples but called as Present after GRP treatment, a high percentage of them were shown to be expressed in the PBMC fraction as well.

In addition, the GRP treatment was shown to be highly reproducible. CV analysis performed across various individuals demonstrated that GRP partially addressed the issue of increased variability associated with utilizing whole blood for expression profiling.

Modeling studies demonstrated a link between large amounts of globin mRNA and reduced sensitivity on GeneChip® arrays. One of the key elements for the successful reduction of globin mRNA, was the design of the Globin Reduction Oligos. Several sequences were initially tested for each of the globin genes during the course of this study. While the provided oligonucleotide sequences were shown to produce the most reliable results, a more comprehensive study may provide additional enhancement to the technique.

The current protocol makes use of specific RNase H digestion to reduce the amount of globin mRNA in the subsequent target preparation. While this approach was shown to work, it is conceivable that other approaches may also be able to bring about the removal of globin transcripts. Direct hybrid capture would allow the depletion step to occur without having to address any issues of non-specific or unwanted nuclease activity from RNase H. Future experimentation will be aimed at simplifying the current strategy in order to provide even greater reproducibility to the method.

CONTRIBUTORS

This study was developed at Affymetrix by Kai Wu, Ph.D. and Garry Miyada, Ph.D. (Product Development). John Martin, Ph.D. and David Finkelstein, Ph.D. (Genomics Collaborations) performed the statistical data analysis. We thank John Palma, Ph.D. (Clinical Applications) for technical input and Raji Pillai, Ph.D. (Genomics Collaborations) and Yan Zhang, Ph.D. (Product Marketing) for helpful discussions. We are also grateful for the blood RNA samples provided by Dr. Chris Clayton and Simon Graham from the Transcriptome Analysis Department of GlaxoSmithKline at Stevenage, UK.

REFERENCE

1. Affymetrix Technical Note *An Analysis of Blood Processing Methods to Prepare Samples for GeneChip® Expression Profiling* (2003).

Appendix: Globin Reduction Protocol

Materials

Reagent Name	Vendor	P/N
Globin Reduction Oligos, HPLC-purified, store at -20˚C α1 5'-TGC AGG AAG GGG AGG AGG GGC TG-3' (nt 512 – 534) α2 5'-TGC AAG GAG GGG AGG AGG GCC CG-3' (nt 512 – 534) β 5'- CCC CAG TTT AGT AGT TGG ACT TAG GG-3' (nt 539 – 564)	Custom Synthesis	N/A
10X Oligo Hyb Buffer, store at -20˚C 100 mM Tris-HCl, pH 7.6 200 mM KCl		N/A
10X RNase H Buffer, store at -20˚C 100 mM Tris-HCl, pH 7.6 10 mM DTT 20 mM $MgCl_2$		N/A
SUPERase • In™, 2,500U	Ambion	2694
RNase H, *E. coli*, 10 U/µL, 200U	Ambion	2292
EDTA, 0.5M	Invitrogen	750009
GeneChip® Sample Cleanup Module	Affymetrix	900371

Procedure

A. Hybridization with Globin Reduction Oligos

1. Prepare the 10X Globin Reduction Oligo Mix with the following final concentration:

Oligo	Final Concentration in 10X Mix
α1	7.5 µM
α2	7.5 µM
β	20 µM

2. Prepare the Hybridization Mix:

Component	Volume	Final Concentration or Amount in Hybridization
Total RNA from whole blood	variable	3 - 10 µg
10X Globin Reduction Oligo Mix	2 µL	2X
10X Oligo Hyb Buffer	1 µL	1X
Nuclease-free Water	variable	to a final volume of 10 µL
Total Volume	**10 µL**	

3. Incubate in a thermal cycler at 70°C for 5 minutes, and then cool to 4°C.
4. Immediately proceed to next step.

B. RNase H Digestion

1. Before starting, dilute an appropriate amount of RNase H (10 U/µL) 10-fold to 1 U/µL with 1X RNase H Buffer.
2. Prepare the RNase H Reaction Mix:

Component	Volume	Final Concentration or Amount in RNase H Digestion
10X RNase H Buffer	2 µL	1X
SUPERase•In	1 µL	
Diluted RNase H (1 U/µL)	2 µL	2 Units
Nuclease-free Water	5 µL	
Total Volume	**10 µL**	

3. Add 10 µL of the RNase H Reaction Mix to each RNA:Globin Reduction Oligo hybridization sample and mix thoroughly.
4. Incubate at 37°C for 10 minutes and cool to 4°C.
5. As soon as the RNase H Digestion is complete, add 1 µL of 0.5M EDTA to each sample to stop the reaction and proceed immediately to the cleanup step.

Important: Do not leave the samples in the RNase H Digestion step at 4°C for an extended period of time. Prolonged incubation may result in undesired nonspecific digestion of the sample.

C. RNase H-Treated Total RNA Cleanup

Use the IVT cRNA Cleanup Spin Column from the GeneChip® Sample Cleanup Module to clean up the RNase H-treated RNA samples. Follow the recommended protocol for the Sample Cleanup Module with the exception of the following steps:

1. Add 80 µL of RNase-free water to the processed sample prior to adding the cRNA Binding Buffer.
2. In the elution step, add 14 µL of RNase-free water to the center of the column, and spin at >= 8,000 x g (>= 10,000 rpm) for 1 minute.
3. Collect the eluate, apply again to the center of column, and spin at >= 8,000 x g (>= 10,000 rpm) for another minute. The final recovery volume after the second spin is approximately 13 µL and the recovery of the cleanup step is ~75%.
4. Use 10-11 µL of the treated total RNA sample in each reaction following the Standard GeneChip Target Labeling Assay as described in the *GeneChip Expression Analysis Technical Manual* (available at www.affymetrix.com) with the exception of (1) using 1 µL of T7-Oligo (dT) Promoter Primer (instead of 2 µL), and (2) extending the IVT labeling reaction to overnight incubation (12-16 hours). Samples may also be stored at -20°C for later use.

Important: Extend the IVT incubation time from 4 hours to overnight (12-16 hours) in order to obtain sufficient material to hybridize onto the GeneChip arrays. With 5 µg of total RNA from PAXgene, more than 30 µg of labeled cRNA is routinely obtained following this protocol.

AFFYMETRIX, INC.

3380 Central Expressway
Santa Clara, CA 95051 USA
Tel: 1-888-DNA-CHIP (1-888-362-2447)
Fax: 1-408-731-5441
sales@affymetrix.com
support@affymetrix.com

www.affymetrix.com

AFFYMETRIX UK Ltd

Voyager, Mercury Park,
Wycombe Lane, Wooburn Green,
High Wycombe HP10 0HH
United Kingdom
Tel: +44 (0) 1628 552550
Fax: +44 (0) 1628 552585
saleseurope@affymetrix.com
supporteurope@affymetrix.com

AFFYMETRIX JAPAN K.K.

Mita NN Bldg., 16 F
4-1-23 Shiba, Minato-ku,
Tokyo 108-0014 Japan
Tel: +81-(0)3-5730-8200
Fax: +81-(0)3-5730-8201
salesjapan@affymetrix.com
supportjapan@affymetrix.com

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