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Technical Note

An Analysis of Blood Processing Methods to Prepare Samples for GeneChip® Expression Profiling

Several different blood processing techniques are currently available to isolate blood cells for RNA purification. These approaches may affect gene expression profiles differently. In order to understand how they impact the sensitivity and variability of expression profiling results with GeneChip® arrays, a systematic analysis was conducted to survey some commonly used techniques. The effects of different processing temperatures, as well as prolonged storage of blood prior to processing, were examined.

Differences in gene expression results were observed using different blood processing methods and are presented in this Technical Note. In addition, incubation of blood overnight before processing was shown to alter the gene expression profiles drastically. Based on these findings, the practical considerations of choosing a blood technique are discussed. Researchers are encouraged to carefully assess which fraction of blood cells is relevant to their research, their microarray assay sensitivity/variability requirements, and the equipment and resource constraints at the site of blood draw in order to make more knowledgeable decisions in selecting blood RNA isolation techniques most suitable for their research needs.

Introduction

Peripheral blood has been an attractive tissue type for biomedical and clinical research, because of its critical role in immune response and metabolism in humans and animal studies, as well as the simplicity and ease of sample collection. Blood is used in biomarker discovery and development of diagnostics in hematological diseases and is also being explored to discover surrogate markers in a wide range of non-hematological disorders. To this end, it is critical that the methods of RNA extraction from blood are effective and efficient.

Many different techniques are used to separate fractions of blood cells prior to RNA isolation, but the impact of these different approaches on genome-wide expression profiling of blood using high-density microarrays has not been well characterized with respect to sensitivity and variability. This Technical Note reports a study which compared commonly used blood isolation and separation protocols, including the PAXgene™ Blood RNA Isolation System, QIAamp® RNA Blood Mini Kits, the Ficoll-Hypaque method (referred to as Ficoll in this Technical Note for simplicity), and BD Vacutainer™-CPT™ Sodium Citrate Tubes. In addition to comparing the gene expression results of different blood processing protocols obtained on the Affymetrix GeneChip® arrays, some variations on the protocols were carried out, such as time delays from blood draw to processing and changes in temperature.

Table 1. Cell types in blood isolated by various separation and fractionation techniques.
*Not shown in this study.

Cell Type	Approximate Number in 1 µL of Blood (Fauci *et al*)	Representative Blood Cell Isolation/Separation Techniques			
		Whole blood: • PAXgene	*Erythrocyte lysis:* • QIAamp	*PBMC:* • Ficoll • BD-CPT	*Specific cells*: • Positive selection • Negative selection
Red blood cells	4.15 – 4.9 million	✓			
Platelets	130,000 – 400,000	✓	✓		
White blood cells Granulocytes: • Neutrophils • Basophils • Eosinophils	4,300 – 10,800 4,500 – 8,300 0 – 20 0 – 700	✓	✓		Any specific subset of cells
Mononuclear cells: • Lymphocytes • Monocytes	 1,600 – 4,500 40 – 100	✓	✓	✓	

As shown in Table 1, several techniques are available for working with blood. These methods include isolation of RNA from whole blood, the selective lysis of erythrocytes prior to RNA isolation, purification of peripheral blood mononuclear cells (PBMC), and separation of specific cell populations based on characteristic cell surface antigens. Cell types predominantly isolated by these techniques and their relative representation in blood (approximate number in 1 µL of blood) are summarized here.

PBMCs are the most transcriptionally active cells in blood. As a result, most studies conducted thus far on blood in many areas of research – such as immunology, infectious and cardiovascular diseases, cancer and biomarker research – have featured the PBMC fraction. This fraction is conventionally isolated by centrifuging whole blood in a liquid density step gradient. It contains lymphocytes and monocytes while excluding red blood cells and granulocytes (eosinophils, basophils, and neutrophils).

Mature red blood cells do not contain RNA but reticulocytes — immature red blood cells — do contain RNA (rRNA, tRNA, and mRNA). The most predominant transcript category in reticulocytes is globin mRNA. Although reticulocytes represent only 0.5 percent to 2.0 percent of the red blood cells in a healthy individual, their RNA may contribute up to 70 percent of total RNA isolated from whole blood due to the very high number of red blood cells present in blood.

The sensitivity and specificity of microarray data can be improved with finer fractionation to eliminate contaminating cell types, such as reticulocytes. As shown in Table 1, different fractionation techniques generate various degrees of homogeneity of the cell types in which researchers are interested. However, as demonstrated in Table 2, the additional cell separation manipulation may require

Table 2. Summary of blood cell fractionation techniques.				
Method	**Equipment for blood processing before total RNA isolation**	**Length of procedure before total RNA isolation**	**Total RNA isolation technique**	**RNA stabilization**
Ficoll	Swinging bucket centrifuge	100 – 150 minutes	TRIzol	No
BD-CPT	Swinging bucket centrifuge	60 – 90 minutes	TRIzol	No
QIAamp	Microcentrifuge	35 – 45 minutes	Column-based purification	No
PAXgene – User developed protocol	N/A	120 minutes	Column-based purification	Yes

immediate processing of blood at the site of blood draw, longer processing time, and additional equipment, all of which may induce *ex vivo* change in expression in certain transcripts.

In order to achieve a balance between the feasibility of performing the additional fractionation of blood cells and the sensitivity and variability requirements of the research, the pros and cons of each technique must be evaluated. Previous studies have been hampered by additional variables associated with individuals, such as gender, age, and health status. In order to minimize these variables, all analyses comparing different techniques were performed in this study on split samples from the same individuals. To also evaluate variables associated with individuals, multiple samples were collected for each method as described in detail in Materials and Methods.

This Technical Note characterizes the impact of different blood separation and isolation techniques on the quality of expression profiling data when used in conjunction with GeneChip microarrays. Direct comparisons of different RNA isolation protocols, as well as various experimental conditions associated with the protocols, are summarized and discussed. The results are presented to help users of GeneChip technology make know-

ledgeable decisions when choosing the most suitable blood and RNA isolation technique for their research purposes.

Results

TOTAL RNA ISOLATION

Sufficient quantities of high-quality RNA are necessary for expression analysis on microarrays. Therefore, quantity and quality of total RNA isolated from blood are important metrics when deciding which blood isolation technique to use. In order to compare Ficoll, BD-CPT, QIAamp, and PAXgene isolation techniques, RNA was isolated as described in Materials and Methods. Multiple samples were collected for each technique as indicated in Figure 1.

Due to some speculation that the manipulation inherent in Ficoll gradient separation may induce changes in the expression profile due to transcriptional activation of PBMC, some researchers opt to perform the gradient separation at lower temperatures to minimize these changes. In order to determine if the temperature of processing affects the quantity and quality of RNA isolated, the Ficoll technique was performed at two different temperatures, either at room temperature (Ficoll RT), or at 8°C (Ficoll 8°C).

Figure 1: Total RNA yield obtained using different blood processing methods. The y-axis shows the total RNA yield and the x-axis shows the technique used for preparation of RNA from blood. The box and whisker plot (Tukey, 1977) represents the interquartile range (between 25% and 75%), and the line within the box denotes the median. The whiskers extend to the last observation before the outliers, which are plotted individually as dots. The table below the figure indicates the number of individuals represented in each method.

Method	Ficoll RT	Ficoll 8°C	Ficoll O/N	BD-CPT	QIAamp	PAXgene
Number of Individuals	15	5	5	10	10	5
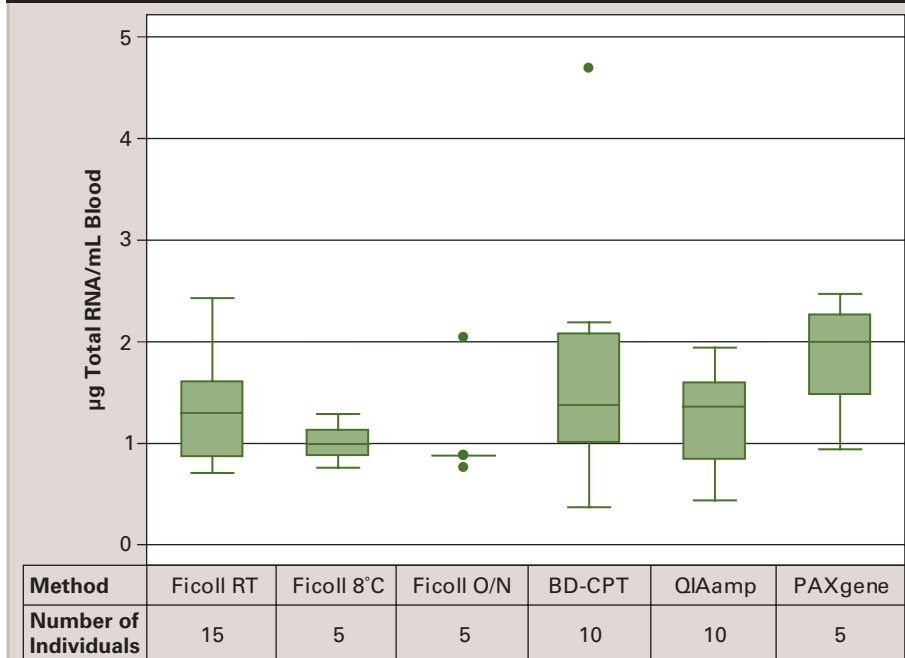
Another variable that can occur in RNA isolation from blood is a time delay between blood draw and processing. Published studies have indicated that when blood is drawn at collection centers and shipped overnight at ambient temperatures, there can be a delay of up to 24 hours from the time blood is drawn until the time it can be processed (de Primo, et al, 2003). To evaluate the effect of this time delay, fractionation was performed immediately after blood draw, as well as after storage of blood for 20 to 22 hours (Ficoll O/N).

As shown in Figure 1, reasonable amounts of total RNA were isolated from all procedures for target labeling for GeneChip expression analysis.

To assess the quality and integrity of the purified total RNA samples, agarose gel electrophoresis was performed on representative samples. As shown in Figure 2, high-quality RNA was obtained from all isolation protocols. In addition, the $OD_{260/280}$ ratios for quantitative RNA quality assessment also met the general requirements (1.9 to 2.1) for proceeding to target labeling for GeneChip array analysis.

The fact that high quality RNA was purified from overnight incubation of blood prior to Ficoll centrifugation was not surprising since the Ficoll gradient itself serves as a screen for live cells. Although there may be cell death occurring during the delay, intact RNA was obtained from the isolated cells.

TARGET LABELING

To evaluate whether the total RNA samples can be processed efficiently using the standard GeneChip target labeling protocol in conjunction with blood isolation techniques, 5 to 15 µg of total RNA were used to generate labeled cRNA targets. As shown in Figure 3, all methods yielded adequate amounts of cRNA for array hybridization as described in the GeneChip Expression Analysis Technical Manual (available at www.affymetrix.com).

Figure 2: Integrity of total RNA samples isolated from different blood processing methods. 1 µg of total RNA isolated by different methods was run on agarose gels. The size marker used was the High Range RNA Ladder from MBI Fermentas. The sizes are shown next to the bands. The RNA obtained was of high quality, as observed by the relative intensities of the 28S and 18S ribosomal bands.
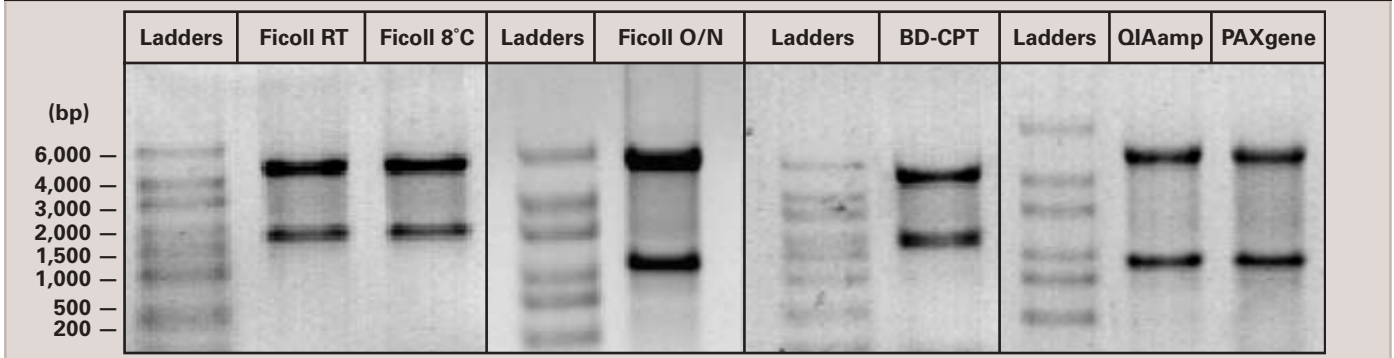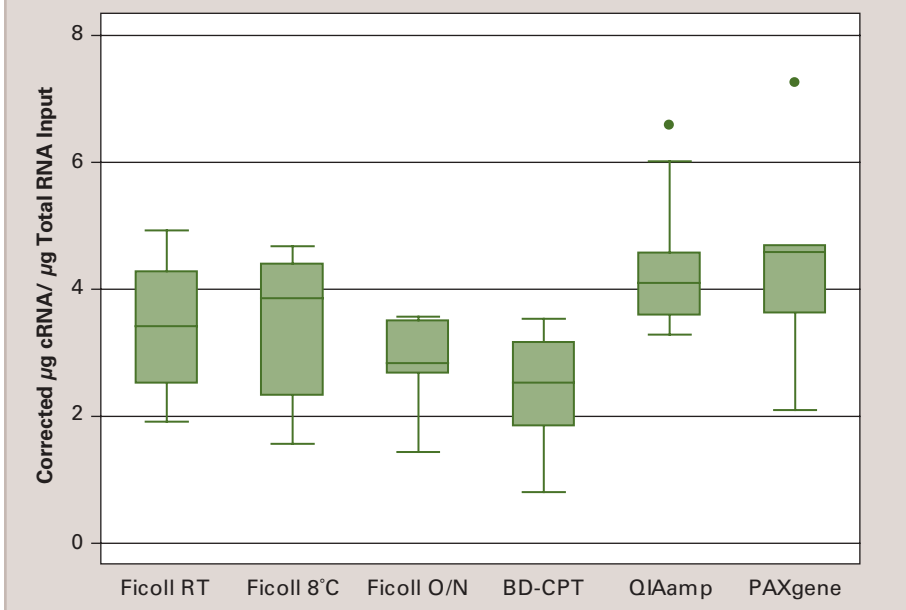
Figure 3: Yield of cRNA targets labeled from samples obtained using different blood processing methods. The box and whisker plot (Tukey, 1977) represents the interquartile range (between 25% and 75%), and the line within the box denotes the median. The whiskers extend to the last observation before the outliers which are plotted individually as dots. The cRNA yield is shown on the y-axis as µg of cRNA obtained per µg total RNA. The x-axis shows the different techniques used for preparation of RNA from blood.

in the 700 bp range (also faintly visable in QIAamp samples) and the relative intensity of the cRNA smear is lower than with other methods. This dominant band is attributed to amplification from globin messages from the red blood cells that are only present in the PAXgene preparations but removed in other methods (Affymetrix, data not shown). The relative reduction in cRNA intensity of the PAXgene samples may result from the competition between the abundant globin messages and the remaining transcripts during amplification and labeling.

GENECHIP® ARRAY QUALITY ASSESSMENT METRICS

After the labeled cRNA targets were hybridized to Affymetrix GeneChip® Human Genome U133A Arrays, the data were analyzed with Affymetrix® Microarray Suite (MAS) 5.0 software. Percent Present calls, RawQ, and Background were used to measure array data quality. Of these metrics, RawQ and Background were determined to be comparable among all methods.

Figure 5 demonstrates the results obtained with Percent Present calls. Although high-quality total RNA was obtained from all methods, consistently lower Percent Present calls are observed in

To assess the length of the cRNA targets, agarose gel electrophoresis was performed on representative samples.

As shown in Figure 4, a typical range of cRNA target lengths (300 bp – 2 kb) was obtained with Ficoll, BD-CPT tubes, and QIAamp. The Ficoll O/N samples (not shown) also generated cRNA targets comparable to other Ficoll methods. However, with PAXgene, a dominant band is observed

Figure 4: Labeled cRNA. 1 µg of cRNA was run on agarose gels before and after the fragmentation reaction. The size marker used was the High Range RNA Ladder from MBI Fermentas. The sizes are shown next to the bands.
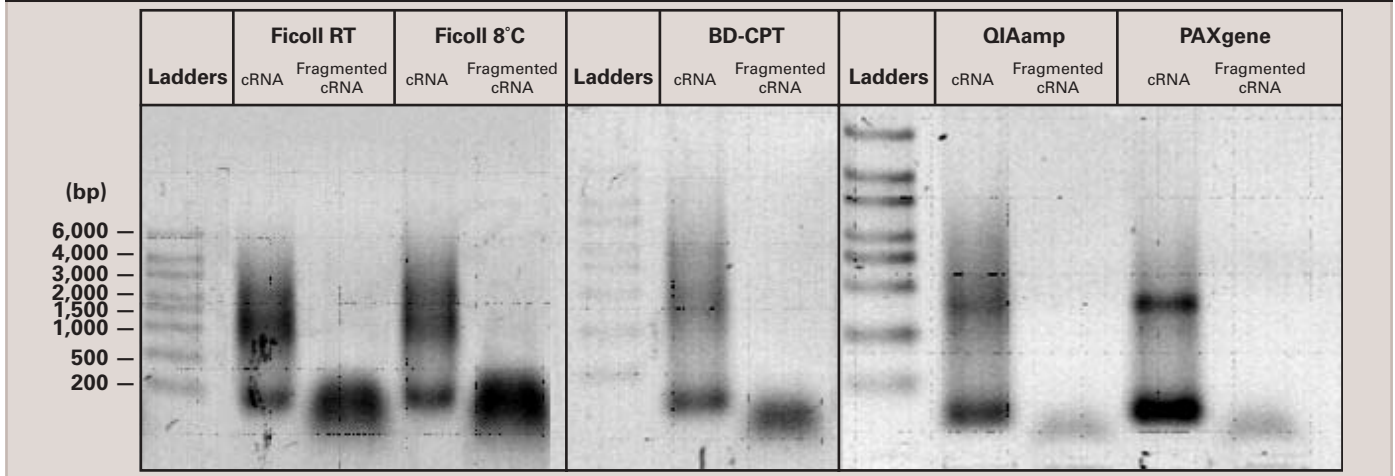
Figure 5: Percent Present calls from arrays hybridized to samples prepared by several techniques. The box and whisker plot (Tukey, 1977) represents the interquartile range (between 25% and 75%), and the line within the box denotes the median. The whiskers extend to the last observation before the outliers which are plotted individually as dots. The y-axis shows Percent Present calls and the x-axis shows the method used.
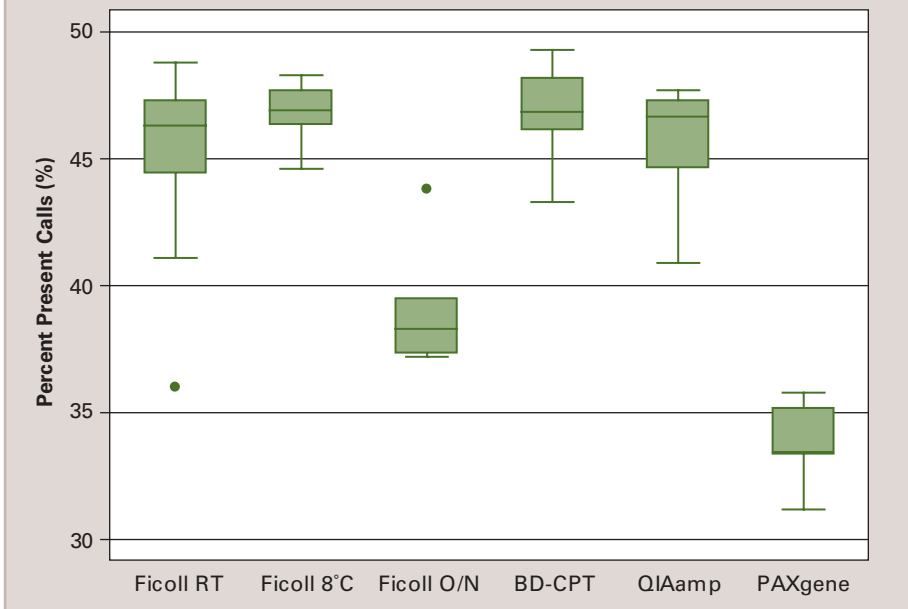
IMPACT ON EXPRESSION PROFILES – FICOLL CENTRIFUGATION PROCESSING TEMPERATURE

For this direct comparison, blood was drawn from each of the five volunteers into citrate tubes. Each blood sample was then split at this stage to be processed by Ficoll centrifugation, either at room temperature or at 8°C. FACS analysis was then conducted on the isolated PBMC fractions to compare the variability and efficiency of the separation methods. The results indicated that there was no significant difference in cell distribution in the PBMC fractions obtained either after processing at room temperature or 8°C (Figure 6). The analysis of T cell subsets (CD4$^+$, CD8$^+$, CD25$^+$, CD69$^+$) also did not reveal any difference (not shown).

Total RNA was then isolated from individual PBMC fractions. Labeled cRNA targets were generated following the standard GeneChip® Eukaryotic Labeling Assay and hybridized to

the PAXgene and the Ficoll O/N samples. These data indicate that whole blood preparation (PAXgene) or delays between blood draw and processing time (Ficoll O/N) can affect the expression results. The reduced sensitivity seen in the PAXgene experiment may be due to the presence of the dominant band in the amplified cRNA target (Figure 4), contributed by the red blood cells in the whole blood RNA preparation.

GAPDH and Actin 3'/5' ratios were used to assess the extent of RNA sample degradation and the efficiency of the target labeling reaction. With all protocols, both ratios were under 3, indicating that the sample integrity was maintained.

Figure 6: FACS analysis of PBMC samples. The major cell subtypes are shown here: CD3$^+$ T cells, CD19$^+$ and CD20$^+$ B cells, CD16$^+$ and CD56$^+$ NK cells, and CD14$^+$ monocytes. Ficoll fractionation was either performed at room temperature (RT) or at 8°C (8°C). The range (5% to 95%), median, and standard deviations are shown.
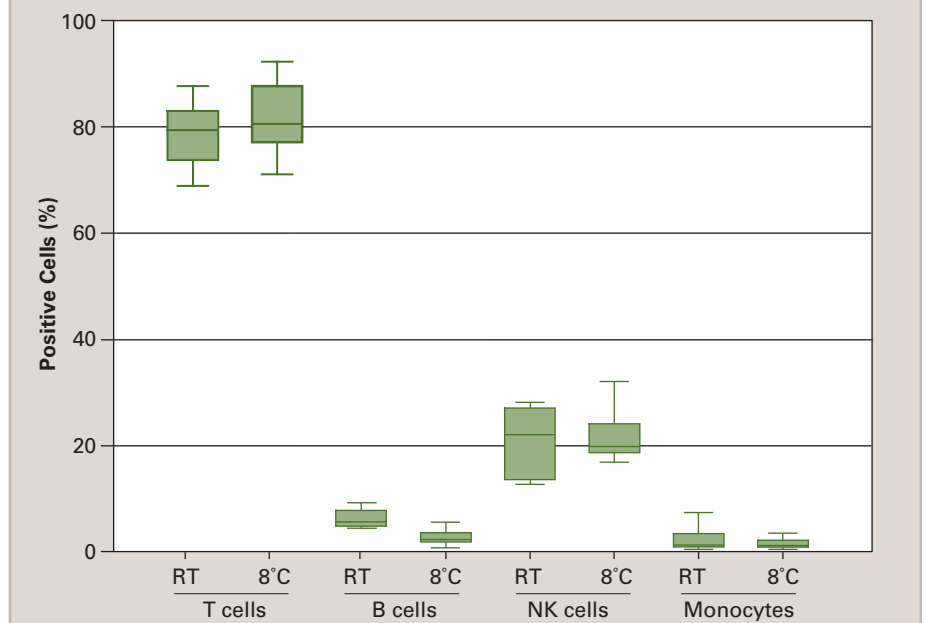
Figure 7: Comparison of magnitude vs. significance of change for samples processed at different temperatures on the Ficoll gradient. The y-axis represents the magnitude of change, comparing processing at 8°C to processing at room temperature (baseline for comparison). It is the $\log_2$ ratio of the geometric means which were computed from (Signal + 50). On the y-axis, 0 represents no change; the two horizontal lines at +1 and -1 represent probe sets that are expressed 2-fold higher or 2-fold lower respectively, at 8°C vs. room temperature. The x-axis represents the significance of change, and plots the *p*-score, defined here as the negative log of the *p*-value from the two-sample *t*-test. A higher *p*-score indicates higher statistical significance of change. The vertical line represents the Bonferroni 95% significance cutoff. Probe sets to the right of the vertical line denote significant differences between two methods.
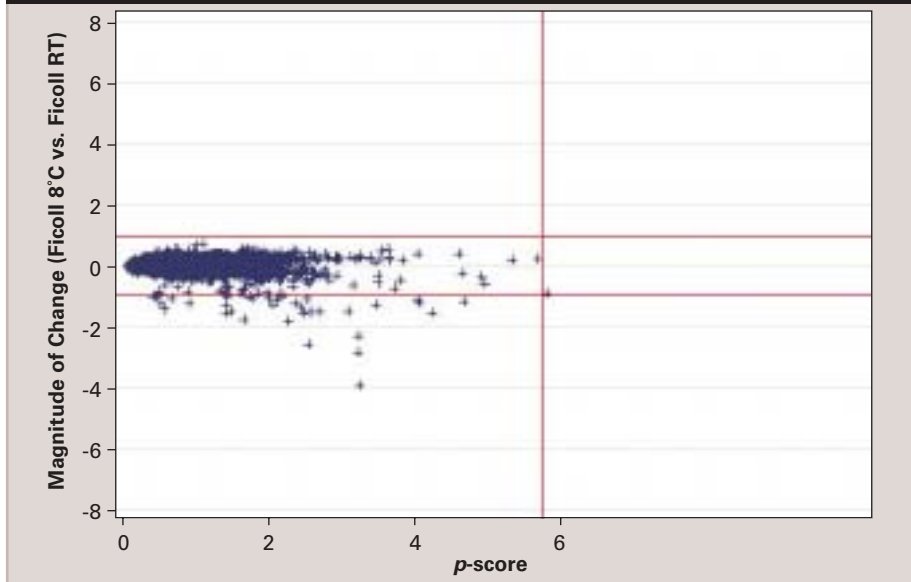
probe set. As a result, the probe sets that show significant difference in expression based on either criterion can be clearly visualized.

As seen in Figure 7, the majority of probe sets showed less than a two-fold change. Only one probe set demonstrated statistical significance according to the Bonferroni test at the 95% confidence level (see Appendix 2 (www.affymetrix.com/support/technical/technotes/bloodappendix_technote.pdf) for description of this probe set). Based on this study, while it is possible that processing at the two different temperatures may induce some change in gene expression, these changes are likely to be subtle or rare.

IMPACT ON EXPRESSION PROFILES – DELAY IN BLOOD PROCESSING ON FICOLL GRADIENT

To measure the effect of a time delay from blood draw to processing, blood was drawn from five individuals into citrate tubes. Each blood sample was then split into two and processed either immediately by Ficoll centrifugation, or after storage

HG-U133A arrays. A two-sample *t*-test was used to identify the genes that showed differential expression between these two methods.

In theory, between two samples, the magnitude of change in expresson levels for the same probe set correlates to the statistical significance of change. However, in practice, due to the unpredictable experimental noise and biological variables, the magnitude of change may become disconnected from the statistical significance value of change. In this case, false positives may be selected whenever magnitude of change is the sole criterion. For example, probe sets with a large magnitude of change may not also be significantly changed by statistical criteria.

The results are displayed with a volcano plot (see Figure 7). This type of plot (Russ Wolfinger, SAS) displays both the magnitude of change and the statistical significance of the change for each

Figure 8: FACS analysis of PBMC samples. The major cell subtypes are shown here: CD3+ T cells, CD19+ and CD20+ B cells, CD16+ and CD56+ NK cells, and CD14+ monocytes. Ficoll fractionation was performed after immediate isolation of PBMC (ASAP) or after overnight (O/N) storage of blood sample (20-22 hours) and subsequent isolation of PBMC. The range (5% to 95%), median, and standard deviations are shown. The asterisk indicates a significant change (*p*<0.05).
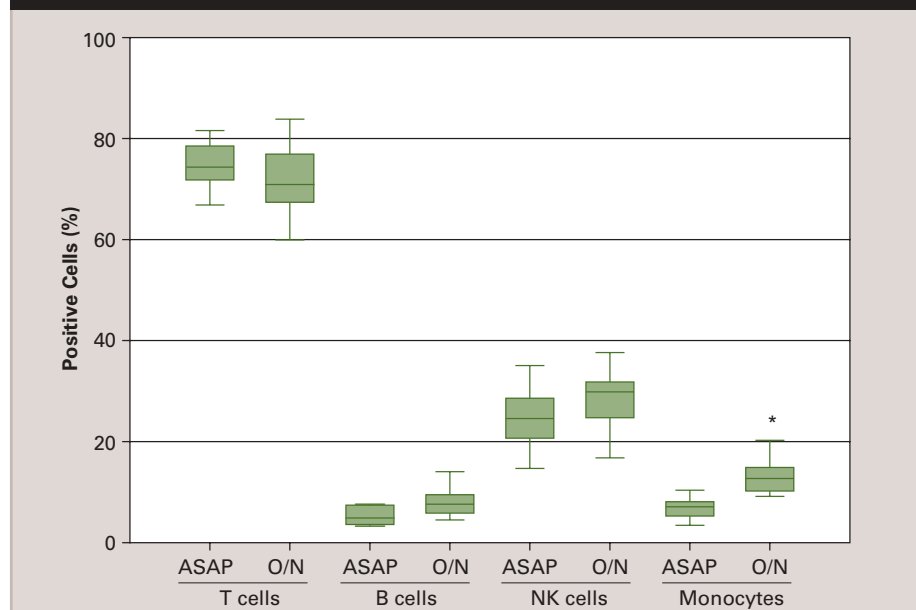
Figure 9: Comparison of magnitude vs. significance of change for samples processed on the Ficoll gradient after different delays. The y-axis represents the magnitude of change, comparing samples processed after overnight storage to those prepared immediately (baseline for comparison). It is the $\log_2$ ratio of the geometric means which were computed from (Signal + 50). On the y-axis, 0 represents no change; the two horizontal lines at +1 and -1 represent probe sets that are expressed 2-fold higher or 2-fold lower, respectively, in samples stored overnight vs. samples processed immediately. The x-axis represents the significance of change, and plots the *p*-score, defined here as the negative log of the *p*-value from the two-sample *t*-test. A higher *p*-score indicates higher statistical significance of change. The vertical line represents the Bonferroni 95% significance cutoff. Probe sets to the right of the vertical line denote significant differences between two methods.
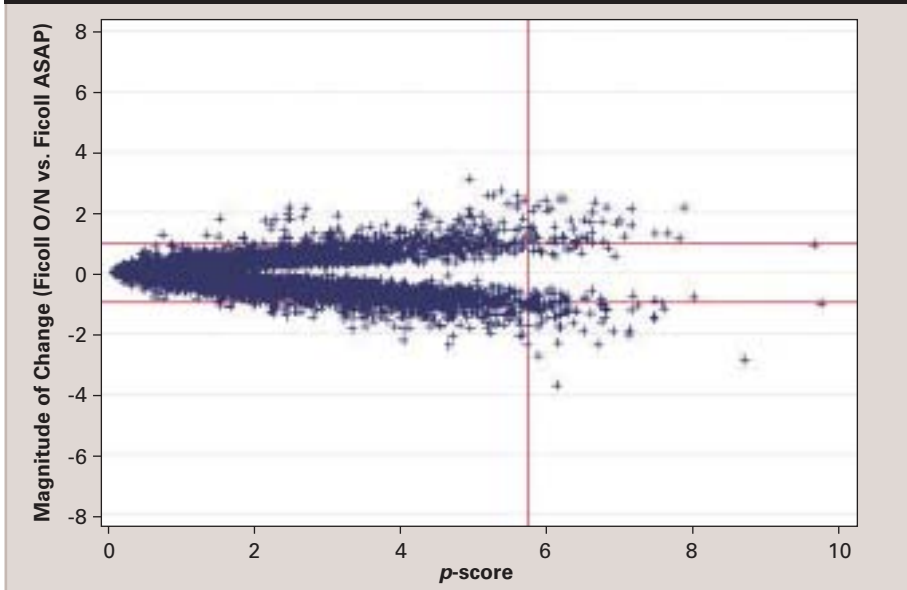
overnight (20-22 hours) at room temperature. FACS analysis was performed immediately after isolation of PBMC at either time point. The results are shown in Figure 8. Except for a relative increase in the proportion of monocytes, there was no striking difference between PBMC isolated immediately after blood draw and blood stored overnight. The analysis of T cell subsets revealed a slightly greater range in the CD4$^+$ T cell subset after overnight storage (data not shown). Comparing other T cell subsets (CD8$^+$, CD25$^+$, CD69$^+$) demonstrated no differences between the two approaches.

Total RNA was then isolated from individual PBMC fractions. Labeled cRNA was prepared and hybridized onto HG-U133A arrays. As shown previously, reduced Percent Present calls were obtained from samples stored overnight (Figure 5). A two-sample *t*-test was per-

formed to identify probe sets that were expressed differently in each method. The results are plotted in Figure 9.

A comparison of the two processing times used in the Ficoll protocol reveals global changes in the resulting gene expression profiles. 150 probe sets showed significant difference in expression, with 90 expressed at a higher level in the samples that were prepared immediately and 60 probe sets expressed at a higher level in the O/N samples. Preliminary GO analyses of these genes on the NetAffx™ Analysis Center (www.affymetrix.com) suggest that genes involved in cellular metabolism are down-regulated after O/N storage. The probe sets are listed in Appendix 3 (www.affymetrix.com/support/technical/technotes/bloodappendix_technote.pdf).

As assessed by gel electrophoresis, general RNA degradation was not detected after overnight incubation (Figure 2). The

cell composition as observed by FACS analysis is generally comparable (Figure 8). Further analysis is necessary to better understand the mechanisms for the observed differences in gene expression. Nevertheless, these results clearly demonstrate that prolonged incubation of the blood samples prior to processing significantly alters array results, and may complicate interpretation.

IMPACT ON EXPRESSION PROFILES - FICOLL CENTRIFUGATION VS BD-CPT VS. QIAAMP PROCESSING

To evaluate the differences between RNA isolation techniques, a direct comparison was performed among Ficoll centrifugation, BD-CPT tube, and QIAamp methods, using blood isolated from the same five individuals. Total RNA was isolated and labeled following the standard GeneChip Eukaryotic Labeling Assay and hybridized onto HG-U133A arrays. Analysis of Variation (ANOVA) was performed to identify the genes that were expressed differentially in the three methods. The results are plotted in Figure 10.

Unlike previous data analyzed using two-sample *t*-tests, this experiment compared data obtained from three different techniques at the same time. For each probe set, a single *p*-score was calculated from the three-way ANOVA analysis, and a higher *p*-score indicates a significant change in expression between at least two of the three samples. Figures 10A and 10B are both plotted against the same *p*-score.

As shown in Figure 10A, a large number of genes were altered between QIAamp and Ficoll methods (see Appendix 4 (www.affymetrix.com/support/technical/technotes/bloodappendix_technote.pdf) for the list of probe sets). 287 probe sets were expressed at a higher level in QIAamp samples, compared with 26 probe sets that were present at a higher level in Ficoll. This may be due to the fact that more cell types were isolated using QIAamp than with Ficoll. Similar results were also obtained comparing QIAamp and BD-CPT samples (not shown). In contrast,

Figure 10: Comparison of magnitude vs. significance of change for samples processed with BD-CPT Ficoll centrifugation, or QIAamp. **A**. The y-axis represents the magnitude of change, comparing QIAamp to Ficoll (baseline for comparison). It is the $\log_2$ ratio of the geometric means which were computed from (Signal + 50). On the y-axis, 0 represents no change; the two horizontal lines at +1 and -1 represent probe sets that are expressed 2-fold higher or 2-fold lower, respectively, in QIAamp samples vs. Ficoll samples. The x-axis represents the significance of change, and plots the *p*-score for ANOVA, derived by taking the negative log of the ANOVA *p*-value. **B**. The axes are as described above, except the y-axis shows the magnitude of change comparing BD-CPT to Ficoll (baseline for comparison).
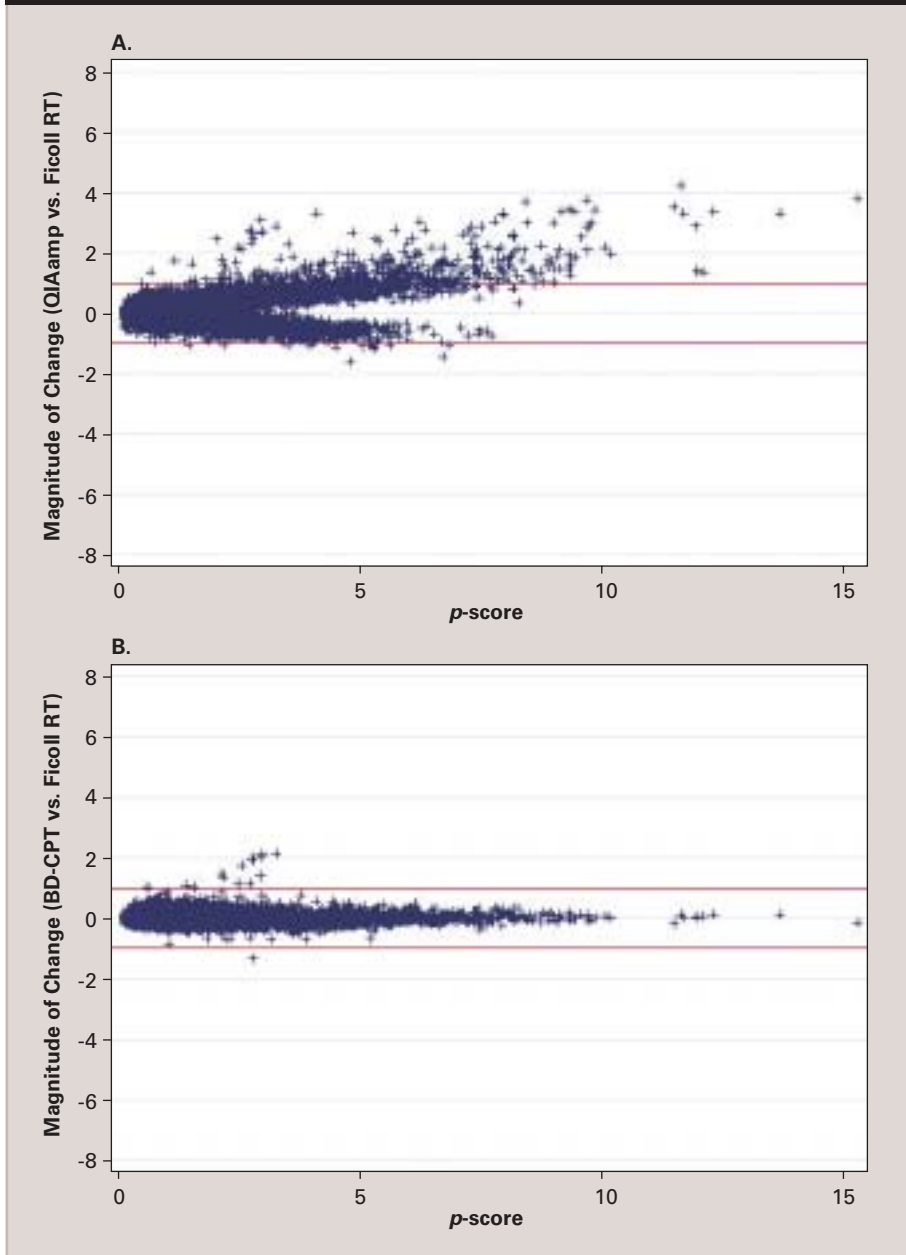
in Figure 10B, very few differences are observed between BD-CPT and Ficoll.

A set of signature genes was selected for red blood cells, granulocytes, and mononuclear cells, as described in Materials and Methods, and are listed in Appendix 1 (www.affymetrix.com/ support/ technical/technotes/bloodappendix_technote.pdf). Expression of these signature genes was analyzed. The results are shown in Figure 11.

Figures 11A and 11B show that the granulocyte markers (shown in blue) are more highly expressed in the QIAamp samples. This is consistent with the fact that QIAamp preparations retain this cellular fraction, whereas BD-CPT and Ficoll preparations exclude it. The mononuclear cell markers (shown in green) show greater expression in the BD-CPT and Ficoll samples, consistent with their selective enrichment by these procedures. Red blood cell markers, shown in red, also appear to be more highly expressed in the QIAamp samples, suggesting that the erythrocyte lysis procedure may not completely remove red blood cells.

Figure 11C shows that mononuclear cells are expressed similarly in both Ficoll and BD-CPT, but some red blood cell markers (shown in red) are expressed more in the BD-CPT samples. When cell fractions were collected, a reddish color in the cell pellets was observed in the BD-CPT purified fraction, indicating that some red blood cells were present. This observation is consistent with the molecular findings shown here. Researchers should be aware of this small amount of contamination of red blood cells in the mononuclear cell fraction.

Figures 10 and 11 reveal global changes in expression data among the different blood processing techniques used, as well as differences in cell type signatures. However, it was observed that there was little overlap between the signature genes
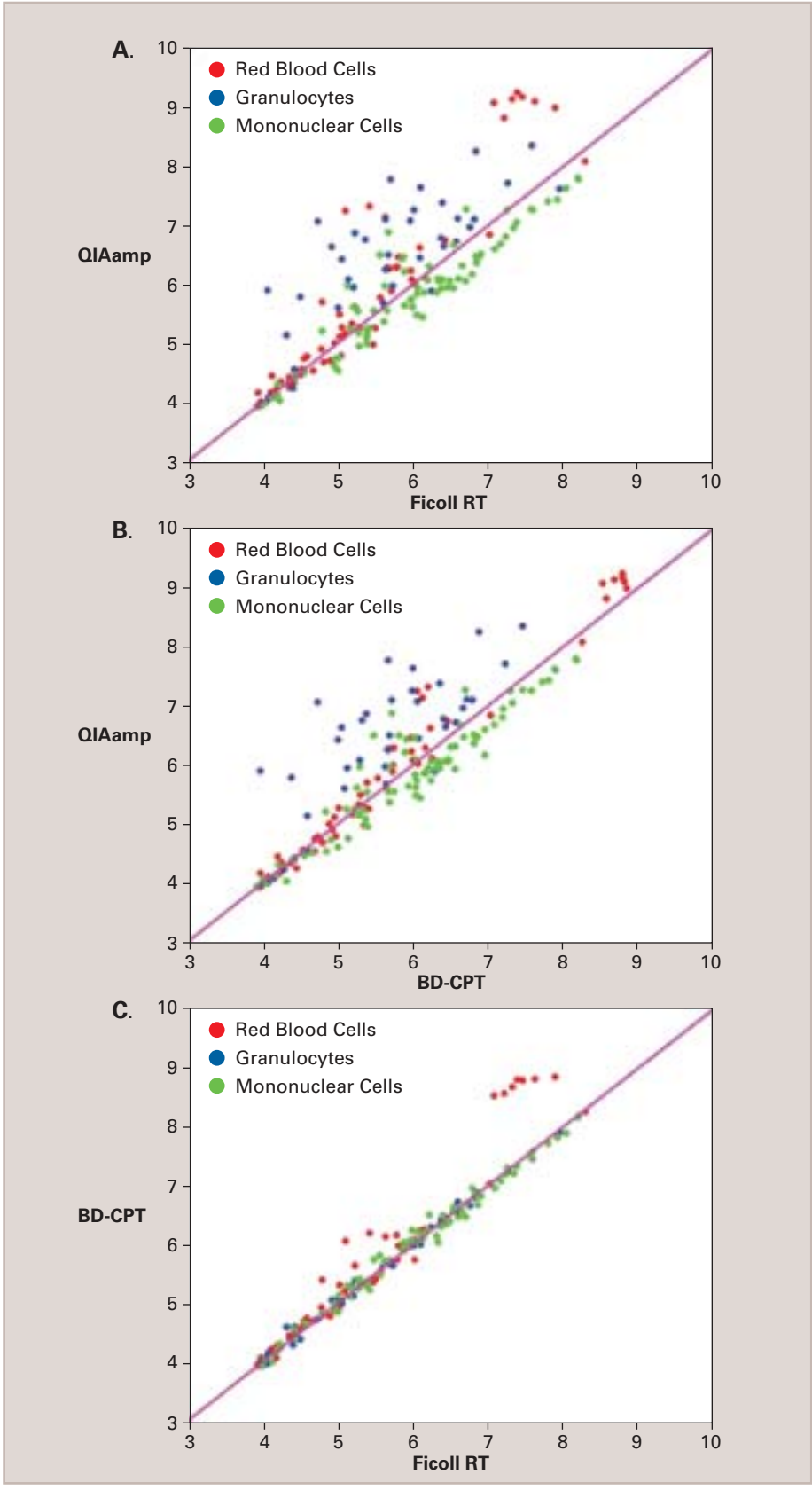
Figure 11: Scatter plots of signature genes in samples from different blood processing methods. Three groups of signature genes are shown and color-coded as indicated. For comparison, the unity line is shown in magenta. Each data point represents the average expression level (n = 5) after natural log transformation, computed as described in the Materials and Methods section. Comparison between two methods is plotted with the expression level of signature genes represented on the two axes in each graph:

A. The y-axis shows signature gene expression levels for QIAamp, and the x-axis for Ficoll.

B. The y-axis shows signature gene expression levels for QIAamp, and the x-axis for BD-CPT.

C. The y-axis shows signature gene expression levels for BD-CPT, and the x-axis for Ficoll.

and the genes considered to be most significantly changed. This could reflect the high stringency of the confidence threshold we used (Bonferroni correction).

IMPACT ON EXPRESSION PROFILES – QIAAMP VS. PAXGENE

To directly compare the two non-PBMC methods, blood from five donors was prepared for GeneChip HG-U133A arrays by either a QIAamp or PAXgene protocol. As shown earlier, PAXgene RNA gave a reduced detection sensitivity on the arrays as determined by the Percent Present calls (Figure 5). A two-sample *t*-test was performed to identify genes that were expressed differently in each method. The results are plotted in Figure 12.

Figure 12 shows that 29 probe sets are significantly changed, of which 22 are present at higher levels in PAXgene and 7 in QIAamp. The differentially expressed genes are listed in Appendix 5 (www.affymetrix.com/support/technical/technotes/bloodappendix_technote.pdf).

Figure 12: Comparison of magnitude vs. significance of change for samples processed with QIAamp or PAXgene. The y-axis represents the magnitude of change, comparing QIAamp to PAXgene (baseline for comparison). It is the $\log_2$ ratio of the geometric means which were computed from (Signal + 50). On the y-axis, 0 represents no change; the two horizontal lines at +1 and -1 represent probe sets that are expressed 2-fold higher or 2-fold lower, respectively, in QIAamp samples vs. PAXgene samples. The x-axis represents the significance of change, and plots the *p*-score, defined here as the negative log of the *p*-value from the two-sample *t*-test. A higher *p*-score indicates higher statistical significance of change. The vertical line represents the Bonferroni 95% significance cutoff. Probe sets to the right of the vertical line denote significant differences between two methods.

processing methods (data set provided by GlaxoSmithKline, Stevenage, UK). Two-sample *t*-test were performed to analyze the differences in the sensitivity and variability.

It was shown previously that reduced sensitivity using RNA isolated from whole blood was observed with respect to Percent Present calls (Figure 5). To evaluate the variability associated with each blood processing techniques, the coefficient of variation (CV%) was examined on a probe set-by-probe set basis to compare the two methods across the same four individuals. Figure 14 represents the CV% plotted against Signal.

As seen in Figure 14, there was greater variability within samples prepared using the PAXgene method than the BD-CPT method. The probe sets with higher variability in PAXgene samples were distributed relatively evenly across the entire range of intensities.
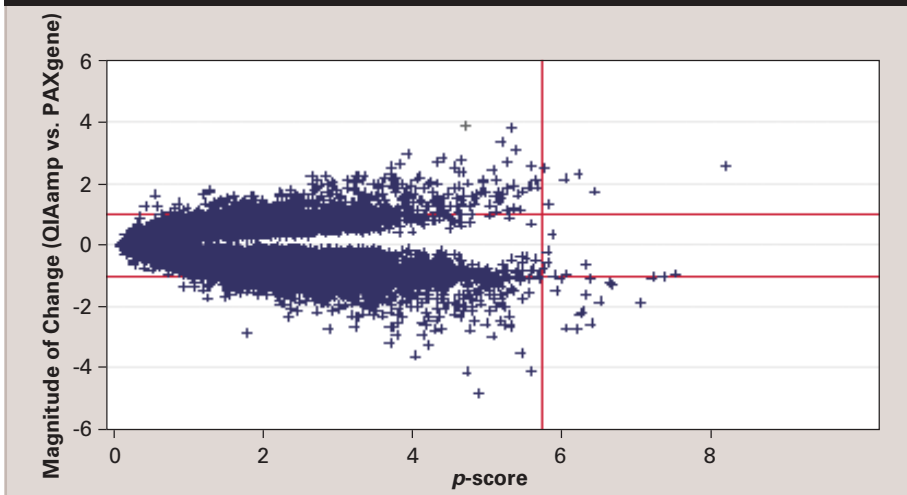
Figure 13 shows the expression of the three groups of signature genes in these two methods. The probe sets which are markers of red blood cells (shown in red) are more abundant in the PAXgene samples compared to QIAamp, confirming that the erythrocyte lysis procedure in QIAamp reduced the red blood cell population in the process. The expression levels for signature genes of granulocytes (shown in blue) and mononuclear cells (shown in green) appear comparable between the two methods, although there may have been a slight increase in expression of these markers in QIAamp samples.

IMPACT ON EXPRESSION PROFILES – PAXGENE VS. BD-CPT

To further define the differences associated with different blood RNA isolation techniques as described in Tables 1 and 2, the variability in results from either the PBMC fraction (BD-CPT) or whole blood (PAXgene) was quantified. In this study, samples were collected from four volunteers and divided for the two

Figure 13: Scatter plot of the signature genes from samples prepared by QIAamp or PAXgene. Three groups of signature genes are shown, and color-coded as indicated. For comparison, the unity line is shown in magenta. Each data point represents the average expression level (n = 5) after natural log transformation, computed as described in the Materials and Methods section. The y-axis shows signature gene expression levels for QIAamp, and the x-axis for PAXgene.
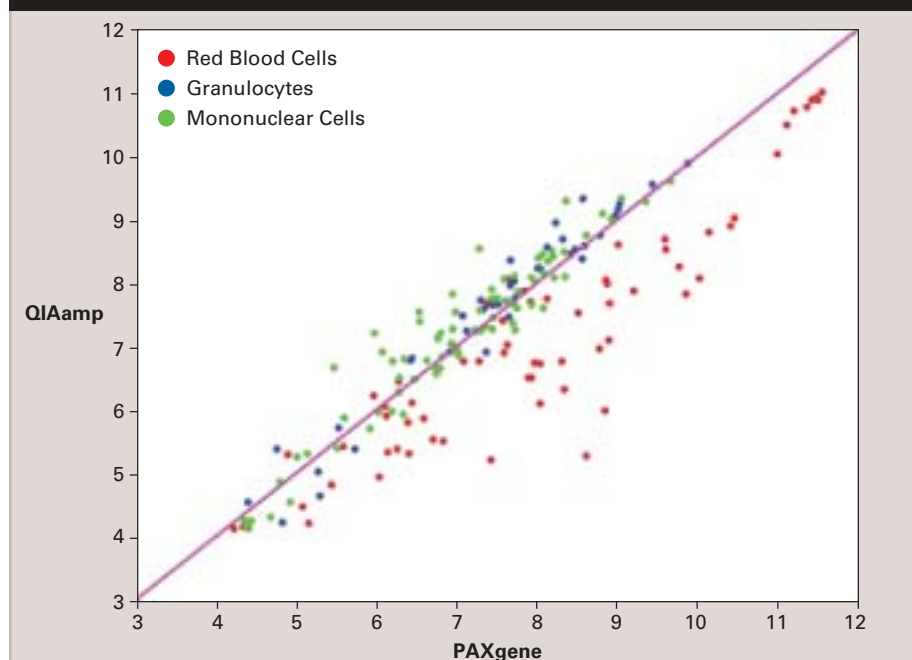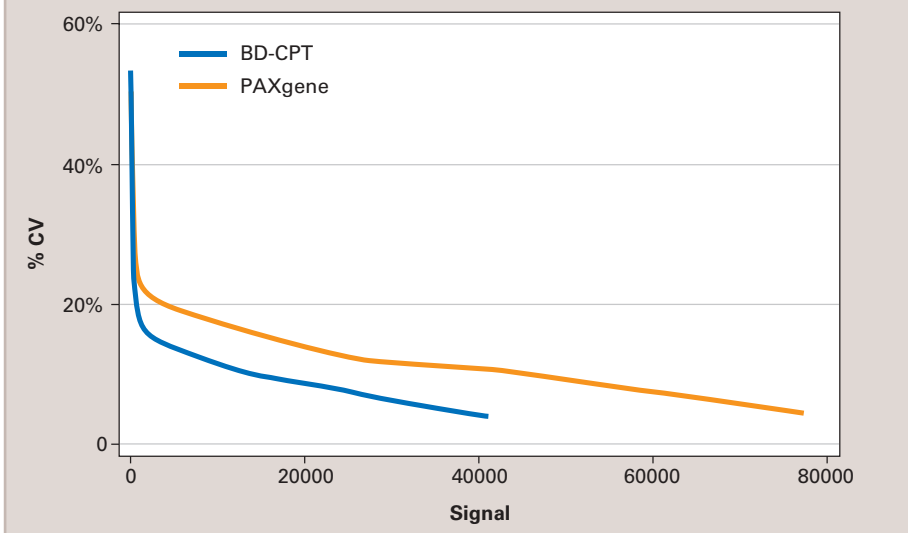
Figure 14: Comparison of variability of BD-CPT and PAXgene. The mean Signal value for each probe set within each method across all four individuals was computed, and the Standard Deviation derived. The CV% (Standard Deviation/Mean) was plotted against the Average Signal for that probe set.

To verify the expression analysis results obtained by the two methods, the expression pattern of signature genes was also analyzed. The results are shown in Figure 16.

The signature genes for cell types only represented in the PAXgene preparation, namely, red blood cells (shown in red), and granulocytes (shown in blue), display drastically lower expression levels in BD-CPT samples. This was expected as BD-CPT samples contain only mononuclear cells while PAXgene samples are obtained from whole blood.

A two-sample *t*-test was used to identify the genes that showed differential expression between these two methods. The results are shown in Figure 15.

A total of 208 probe sets were identified to have Signal values that were significantly different, with 84 and 124 probe sets displaying higher or lower intensity, respectively, in the PAXgene preparation. A complete list of these probe sets is included in Appendix 6 (www.affymetrix.com/support/technical/technotes/bloodappendix_technote.pdf) for reference.

The probe sets displaying higher expression in the PAXgene method may represent the genes expressed in the cell types that are excluded in the BD-CPT preparation. In contrast, the probe sets more highly represented in the BD-CPT method could include low expression genes that are not readily detected in the PAXgene protocol, as well as those genes that may be induced consistently by the *ex vivo* manipulation during BD-CPT manipulation.

Figure 15: Comparison of magnitude vs. significance of change for samples processed with PAXgene or BD-CPT. The y-axis represents the magnitude of change, comparing PAXgene to BD-CPT (baseline for comparison). It is the $\log_2$ ratio of the geometric means which were computed from (Signal + 50). On the y-axis, 0 represents no change; the two horizontal lines at +1 and -1 represent probe sets that are expressed 2-fold higher or 2-fold lower, respectively, in PAXgene samples vs. BD-CPT samples. The x-axis represents the significance of change, and plots the *p*-score, defined here as the negative log of the *p*-value from the two-sample *t*-test. A higher *p*-score indicates higher statistical significance of change. The vertical line represents the Bonferroni 95% significance cutoff. Probe sets to the right of the vertical line denote significant differences between two methods.
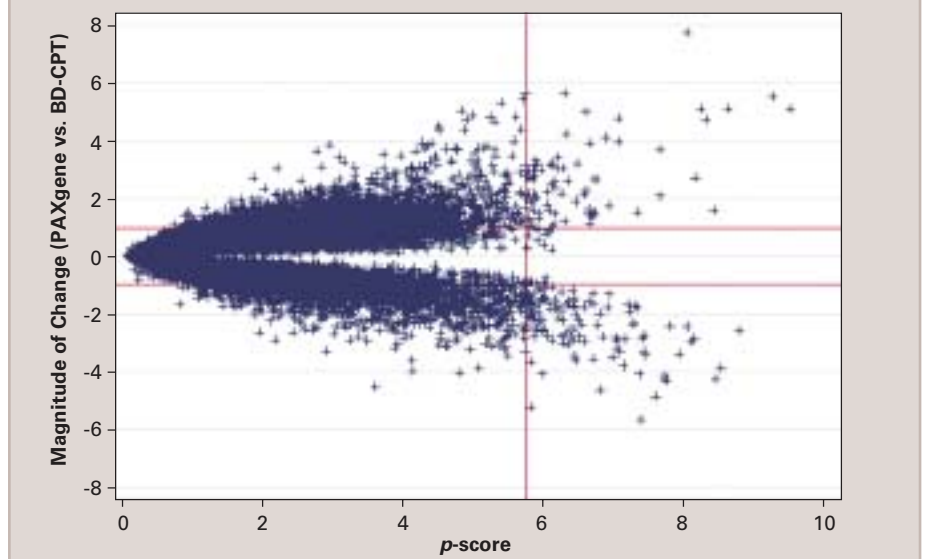
Figure 16: Scatter plot of the signature genes from samples prepared by PAXgene or BD-CPT. Three groups of signature genes are shown and color-coded as indicated. For comparison, the unity line is shown in magenta. Each data point represents the average expression level (n = 4) after natural log transformation, computed as described in the Materials and Methods section. The y-axis shows signature gene expression levels for PAXgene, and the x-axis for BD-CPT.
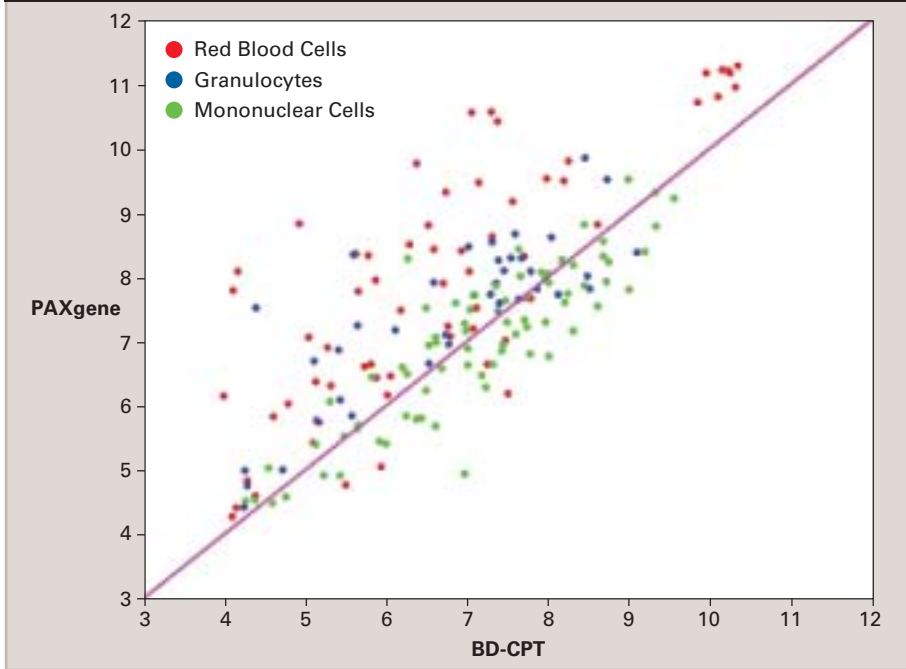
Main Findings

In conclusion, the preceding analyses have revealed the following observations:

- Expression array results were similar for experiments conducted with the Ficoll gradient technique performed at room temperature and 8°C.
- Expression array results were comparable for experiments conducted with either BD-CPT or Ficoll centrifugation methods.
- Many changes in gene expression data were observed when blood was incubated overnight before Ficoll processing.
- QIAamp, Ficoll, and BD-CPT methods generated comparable detection sensitivity for identical samples, whereas the PAXgene method resulted in drastic differences in expression results and detection sensitivity.

Discussion

This Technical Note reports GeneChip array data obtained on RNA samples prepared from various blood processing methods, including the PAXgene, QIAamp, Ficoll, and BD-CPT techniques. Results presented here focus on the impact each method has on expression profiles. This information should provide insight for scientists to help them make an informed decision on which method is most suitable to use for their own research when they are considering various blood protocols in conjunction with GeneChip expression microarrays.

RECOMMENDATIONS

Because each method varies from the others, it is recommended that one single method be used consistently throughout a study in order to obtain meaningful results. Due to the fact that research parameters and experimental conditions may limit the blood isolation protocol choice, scientists should take these variables into account before initiating large studies in order to be able to use the same blood isolation technique consistently throughout.

In addition to the main findings listed above, several recommendations to consider are summarized here:

Storage of Blood Before Processing

Results suggest that storing blood for a prolonged period of time prior to processing negatively affects the results. In this study, an overnight storage scenario before Ficoll preparation was compared to Ficoll preparation conducted immediately after blood was drawn. It was found that the delay in blood processing resulted in significant changes in expression profiles compared with the samples prepared immediately. Therefore, it would be beneficial to reduce the time of storage and transport to minimize the effect, and consequently, improve the quality of experimental results. Ideally, RNA should be isolated immediately after blood samples are drawn, and the frozen RNA samples can then be transported and stored. A time-course study is necessary to assess whether there is a shorter period of time for storage or transport of blood that may not be detrimental to the results.

Points to Consider for Selecting Blood Processing Methods

It is recommended that users carefully evaluate their research requirements, as well as constraints, and use the data presented here to help choose the method that best matches their needs. Some of the basic considerations in selecting a method include:

- Cell types of interest: This may limit the method of choice. For example, if neutrophils are the primary cells being studied, PAXgene and QIAamp are the only options of those discussed in this Technical Note, whereas the PBMC fraction isolated by the BD-CPT or Ficoll methods may not be appropriate.

- Availability of equipment and trained personnel at the site of blood draw: This can be critical since the different methods require varying degrees of blood manipulation following blood draw, ranging from zero processing, in the case of PAXgene, to relatively complex, skilled processing, such as preparation and running the Ficoll gradient.
- Assay sensitivity requirements: Different methods displayed varying levels of sensitivity with the GeneChip microarrays. Depending on research requirements, this factor may be critical. Using a method with relatively compromised sensitivity, such as PAXgene, may be acceptable for some applications. For others it may be beneficial to perform additional fractionation, to isolate only the cells belonging to a subtype.
- Tolerance to increased assay variability: Although not included in this study, it has been speculated that increasing the number of replicates may help reduce variability in general. Therefore, if constrained by other requirements, adequate planning and design of experiments may allow for tolerance of the increased variability observed in some of these methods.

Additional information is provided in this Technical Note, such as the list of signature genes, as well as those that were documented in our study to be significantly different using various methods. Although detailed analyses have not been performed on the expression pattern of all of the genes listed, this information may be used as a reference by users for comparison purposes in the initial assessment of the quality of their data.

Contributors

This study was performed at the Molecular Tumor Biology and Tumor Immunology Unit, University of Cologne, Germany, by Dr. Svenja Debey, Ulrike Diening, and Prof. Joachim L. Schultze, in collaboration with Affymetrix Genomics Collaborations (Dr. Raji Pillai), Data AnalysisTeam (Dr. David Finkelstein and Dr. John Martin), and Affymetrix Product Marketing (Dr. Yan Zhang-Klompus).

The data from the PAXgene – BD-CPT study was contributed by Dr. Chris Clayton and Simon Graham, Transcriptome Analysis Department, GlaxoSmithKline, Stevenage, U.K., as was a list of cell type-specific genes. We thank Dr. Gavin Sherlock of the Stanford Microarray Center and Dr. Alan Williams of the Affymetrix Bioinformatics Department for assistance with mapping the IMAGE clones from Whitney *et al* to the HG-U133 arrays, and Dr. Michael Morrissey of Millennium Pharmaceuticals, Cambridge, Massachusetts, U.S.A. for the list of probe sets considered to be monocyte-specific.

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Material and Methods

BLOOD CELL FRACTIONATION AND TOTAL RNA ISOLATION TECHNIQUES

Blood was collected from healthy individuals after informed consent following the institutional review board at the University of Cologne.

- Ficoll-Hypaque – Citrate blood collection tubes were used to collect 50 mL of peripheral venous blood from each healthy volunteer. The blood samples were then transported within 15 minutes to the laboratory and either stored for the lengths of time indicated in the Results section, or processed immediately. Ficoll-Hypaque density centrifugation was performed following standard methodology. The samples were divided into two 25 mL aliquots and centrifuged at 400 x g for 7 minutes at either 18 to 20°C (room temperature, RT) or 8°C. The upper plasma phase was removed and the rest of the sample was diluted with an equal volume of 1X PBS and mixed by pipetting. The Ficoll gradient centrifugation was then performed either at room temperature or at 8°C. For the 8°C samples, the centrifuge, PBS, and Ficoll were cooled down 30 minutes prior to use. The diluted blood samples were overlaid on 12.5 mL of Ficoll and centrifuged at 800 x g for 25 minutes without brake. The interphase was then transferred to 30 mL of 1X PBS and mixed by inverting the tubes, and centrifuged at 500 x g for 10 minutes with brake. The supernatant was discarded and the cell pellet was resuspended in 20 mL of 1X PBS by pipetting. As determined by trypan blue staining, for all experiments, over 95% of the cells obtained were viable. The cells were pelleted by centrifugation at 500 x g for 5 minutes with brake and the supernatant was removed completely.

One mL of the TRIzol® Reagents (Invitrogen, CA, USA) was added to every 1 x 10⁷ cells and the cells were lysed by repetitive pipetting and incubated for 5 minutes at ambient temperature to permit complete dissociation of nucleoprotein complexes. The samples were stored at -80°C until total RNA isolation (for details, see the RNA Isolation section below).

- BD-CPT – BD Vacutainer™ CPT™ Sodium Citrate Tubes (Becton Dickenson, NJ, USA) were used to collect peripheral venous blood from healthy volunteers. Three tubes were collected from each individual with 8 mL of blood in each tube. The blood samples were mixed by gently inverting the tubes 5 times, prior to transport to the laboratory for processing. The transition was kept as short as possible (up to 15 minutes) and the tubes were kept upright at ambient temperature. Immediately before centrifugation, the blood samples were remixed by inverting the tubes 8 to 10 times. Centrifugation was performed at 1,650 x g for 20 minutes in a swinging bucket centrifuge at room temperature (18°C) with brake. The upper layer was transferred to a new tube containing 30 mL of 1X PBS. Cells were centrifuged at 500 x g for 10 minutes with brake and supernatant was discarded. The cell pellet was resuspended in 20 mL of 1X PBS. As indicated by Trypan Blue exclusion test, cell viability was always >95%. The cells were centrifuged again at 500 x g for 5 minutes with brake and the supernatant was removed. One mL of the TRIzol® Reagents (Invitrogen, CA, USA) was added to every 1 x 10⁷ cells and the cells were lysed by repetitive pipetting and incubated for 5 minutes at ambient temperature to permit complete dissociation of nucleoprotein complexes. The samples were stored at -80°C until total RNA isolation (for details, see the RNA Isolation section below).

- RNA Isolation – After thawing, 0.2 mL of chloroform per mL of the TRIzol® Reagents was added to each sample. The tubes were shaken by hand for 15 seconds and incubated for 3 minutes at room temperature. The samples were then centrifuged at 13,000 rpm in a microcentrifuge for 10 minutes at 4°C. Following centrifugation, the upper aqueous phase was transferred to a fresh tube and the RNA was precipitated by the addition of 0.5 mL of isopropanol per mL of the TRIzol Reagent used in the initial homogenization. The samples were incubated for 10 minutes at room temperature and then centrifuged at 13,000 rpm in a microcentrifuge for 30 minutes at 4°C. The supernatant was removed and the RNA pellets were washed twice by adding 1 mL of 80% ethanol, mixed by vortexing and centrifuged at 13,000 rpm for 5 minutes at 4°C. After washing, the RNA pellets were air-dried for 5-10 minutes, dissolved in RNAase-free water and incubated for 5 to 10 minutes at 55°C. The RNA was purified with the RNeasy MinElute Cleanup Kit (QIAGEN GmbH, Germany).

- PAXgene™ Blood RNA Isolation System – Three PAXgene Blood RNA Tubes (QIAGEN GmbH, Germany) with 2.5 mL blood in each tube were used to collect peripheral venous blood from each healthy volunteer following the manufacturer's recommended procedure, including the optional DNase digestion step. The RNA from the three tubes for each individual was pooled prior to quantitation. The RNA was then concentrated with the RNeasy MinElute Cleanup Kit (QIAGEN GmbH, Germany).

- QIAamp® RNA Blood Mini Kits – Citrate blood collection tubes were used to collect peripheral venous blood from healthy volunteers. 9 to 18 mL of blood samples were divided into 1.5 mL aliquots or less. No more than 1 x 10⁷ cells were loaded on one column. The

samples were processed individually with the QIAamp RNA Blood Mini Kits (QIAGEN GmbH, Germany) following the manufacturer's recommended procedure including the optional DNase digestion step. RNA was pooled prior to quantitation. The RNA was then concentrated with the RNeasy MinElute Cleanup Kit (QIAGEN GmbH, Germany).

FLOW CYTOMETRY

Cell phenotype was defined by four-color staining performed on PBMCs using the following antibodies: FITC conjugated anti-CD3, -CD71, -CD45RA (Pharmingen), PE-conjugated anti-CD4 (Pharmingen), PerCP-conjugated anti-CD19, -CD20, -CD8 (Becton Dickinson), APC-conjugated anti-CD14, -CD69, -CD25, -CD45RO (Pharmingen), Simultest anti-CD3/16+56 (Becton-Dickinson), and corresponding mouse IgG controls: Simultest γ1 γ2a, APC-conjugated anti-γ2a (Becton Dickinson), PerCP-conjugated γ1 (Pharmingen).

In brief, cells were washed with 2 mL cell wash (Becton Dickinson) and centrifuged for 5 minutes at 450 x g with brake. Supernatant was removed and the cells were resuspended in 100 uL cell wash and stained for 20 minutes at 4°C with the appropriate antibodies. After washing, cells were fixed with cell wash containing 2% (v/v) formaldehyde. Samples were run on a FACS Calibur (Becton Dickinson) and analyzed with CellQuest 3.3 software.

GENECHIP® TARGET LABELING AND ARRAY HYBRIDIZATION

Total RNA (5-20 μg) obtained from each sample was labeled following the standard target labeling protocol as described in the GeneChip® Expression Analysis Technical Manual. The amount of cRNA obtained from the *in vitro* transcription reaction was quantified using a spectrophotometer. Following fragmentation, 10 μg of cRNA target were hybridized to HG-U133A arrays.

GENECHIP ARRAY DATA STATISTICAL ANALYSIS

The array Signal values from MAS 5.0 were normalized to a target intensity of 100 (in most cases, scaling factor was < 3. In some of the PAXgene or QIAamp samples, the scaling factor is between 5 and 6). Then, the value 50 (roughly twice the Standard Deviation of the Background) was added to all measurements. This serves to stabilize the variance of Signal and Background near the low end of expression, and has very little effect on the high end of data (Affymetrix Inc, unpublished observations). After transformation, the data more closely conform to the assumptions that underlie t-test and ANOVA analyses. Alternatives to variance stabilizing approaches may also be used, such as filtering based on Detection Calls.

Following this procedure, data were subjected to a natural log transformation so that each data point has more equivalent influence on the final outcome.

When results from two RNA isolation methods were compared where the blood samples were obtained from the same five individuals, a two-sample t-test with equal variances was used.

When results from three methods were compared with blood from the same five individuals, ANOVA analysis was performed.

All these analytical methods identified genes that were changed among the different blood preparation methods. These genes vary in terms of magnitude of change, as well as statistical significance of change. Either criterion may be used as a filter to select genes for further analyses. Genes selected by magnitude of change may include those that do not show consistent change. Conversely, genes selected based on stringent statistical cut-off may include some genes that show very subtle changes.

We selected a statistical approach, namely the Bonferroni correction, as a threshold.

This stringent approach selects only genes that can be considered true positives, with no false positives present (95% confidence). The disadvantage of this approach is that in requiring no false positives, we fail to select some authentic changes, i.e., we generate some false negatives. Less stringent thresholds may be used, for example, those based on the False Discovery Rate (FDR, Yoav Benjamini, Tel Aviv University).

These analyses were performed using the STATA/S.E. 8.0 software package (College Station, Texas, U.S.A) and the MATLAB 6.5 software package (The MathWorks, Natick, Massachusetts, U.S.A).

GENERATION OF SIGNATURE GENES FOR DIFFERENT BLOOD CELL TYPES

HG-U133A probe sets that were specific to different cell types in blood based on information were identified from several sources listed below:

- 29 genes were found to be expressed in monocytes but demonstrated very little or no expression in other blood cells (Millennium Pharmaceuticals, Cambridge, MA, U.S.A).

- 12 genes were used as markers of different blood cells (GlaxoSmithKline, Stevenage, U.K.). These were originally identified in the GeneChip® Human Genome U95Av2 set of arrays, but were mapped to HG-U133A using the NetAffx™ Analysis Center (www.affymetrix.com).

- A recent publication by Whitney *et al*, (2003) showed seven groups of genes, some classified according to cell type, others according to variability seen among the individuals studied. 797 original IMAGE clone IDs were represented in these seven groups. We identified 604 probe sets on the GeneChip HG-U133 Array Set corresponding to 555 of the

797 IMAGE clones based on IMAGE clone identifiers and GenBank sequence identifiers. These were included in the selection of the signature genes.

- A set of 14 probe sets that represented different forms of hemoglobin on the HG-U133A array were added to the signature list.

The signature genes were compiled from the above sources and a final list was generated based on our ability to map them with confidence to the HG-U133 array set. This list contains 181 probe sets. Of these, 78 probe sets are for mononuclear cells (monocytes and lymphocytes), 38 for granulocytes (neutrophils and eosinophils), and 65 for red blood cells (erythrocytes and reticulocytes). It is important to note that for many of these probe sets (particularly those derived from Whitney *et al*), their expression primarily in the respective cell types has not been confirmed experimentally on GeneChip arrays. They were used here solely for the purpose of cross-validation and verification of analysis, and should be used as reference only. A complete listing of these signature gene IDs on HG-U133A arrays is provided in Appendix 1 (www.affymetrix.com/support/technical/technotes/bloodappendix_technote.pdf).

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