Biomarkers, July–August 2005; 10(4): 310–320

Optimized blood cell profiling method for genomic biomarker discovery using high-density microarray

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Abstract

High-quality biomarkers for disease progression, drug efficacy and toxicity liability are essential for improving the efficiency of drug discovery and development. The identification of drug-activity biomarkers is often limited by access to and the quantity of target tissue. Peripheral blood has increasingly become an attractive alternative to tissue samples from organs as source for biomarker discovery, especially during early clinical studies. However, given the heterogeneous blood cell population, possible artifacts from *ex vivo* activations, and technical difficulties associated with overall performance of the assay, it is challenging to profile peripheral blood cells directly for biomarker discovery. In the present study, Applied BioSystems' blood collection system was evaluated for its ability to isolate RNA suitable for use on the Affymetrix microarray platform. Blood was collected in a TEMPUS tube and RNA extracted using an ABI-6100 semi-automated workstation. Using human and rat whole blood samples, it was demonstrated that the RNA isolated using this approach was stable, of high quality and was suitable for Affymetrix microarray applications. The microarray data were statistically analysed and compared with other blood protocols. Minimal haemoglobin interference with RNA labelling efficiency and chip hybridization was found using the TEMPUS tube and extraction method. The RNA quality, stability and ease of handling requirement make the TEMPUS tube protocol an attractive approach for expression profiling of whole blood to support target and biomarker discovery.

Keywords: *Affymetrix, biomarker, haemoglobin reduction, microarray, peripheral blood mononuclear cell (PBMC), TEMPUS tube, whole blood*

(Received 22 March 2005; accepted 2 June 2005)

Introduction

Biomarkers have become an essential tool for predicting which patients will respond to therapy or help drive key cost-effective decisions during the different stages of drug development. The emerging high content approaches in genomics, proteomics and associated informatics tools are now being applied proactively to biomarker discovery (Ideker et al. 2001, Frank & Hargreaves 2003, Hood et al. 2004). While biomarkers

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ISSN 1354-750X print/ISSN 1366-5804 online © 2005 Taylor & Francis
DOI: 10.1080/13547500500218583

can be in various forms, conventional biomarker strategies often focused on the targeted tissue to monitor disease progression, toxicity and/or drug action. Such targeted approaches are limited, in particular by restricted access to the target tissues, such as the brain. One solution has been to focus on secreted molecules in the hope that the products of the gene identified *in situ* can be translated to surrogates that are readily measurable in peripheral body fluids, such as serum, cerebrospinal fluid or urine. However, due to factors such as dilution effects, dynamic range and non-exclusive expression, this approach is not always effective. Transcript profiling of peripheral blood provides an attractive alternative for biomarker discovery (Twine et al. 2003, Amundson et al. 2004, Xu et al. 2004, Tsuang et al. 2005). However, given the heterogeneous blood cell population, artefacts from *ex vivo* activation of the lymphocytes during cell fractionation, and technical difficulties to isolate and label whole blood RNA, it remains technically challenging to profile peripheral blood cells directly for biomarker discovery (Fan & Hegde 2005). One of the technical hurdles is the overwhelming expression of haemoglobin genes in erythrocytes, which severely interferes with RNA labelling, linearity of RNA amplification and subsequent array hybridization. Utilization of the purified peripheral blood mononuclear cells (PBMC), selective haemoglobin RNA depletion or selective red cell lysis provides possible solutions to this problem (Affymetrix technical report, Santa Clara, CA, USA). Such procedures are costly, labour intensive and might incur undesirable *ex vivo* lymphocyte activation not relevant to disease progression or drug action. The present study applied a rapid method to isolate high-quality RNA from human and rat whole blood using Applied BioSystems' blood collection and RNA extraction platform. It was demonstrated that this system was robust and provided high-quality RNA capable of producing satisfactory chip performance. The microarray data were statistically analysed and compared with those generated using other blood protocols. A reduction of haemoglobin interference was observed with this method. In addition, it was observed that sample freeze–thawing did not decrease RNA yield or affect overall chip performance. The quality, stability and ease of sample handling made this approach suitable for applications using clinical blood samples.

Materials and methods

Blood collection and RNA isolation

Blood from healthy human donors and normal healthy male adult rats were used. A total of 3 ml whole blood were collected into the TEMPUS Blood RNA tubes (ABI, Foster City, CA, USA) containing 6 ml Applied BioSystems' RNA stabilization reagent. The tubes were mixed vigorously for 30 s to ensure complete mixture of the stabilizing reagent and the blood. The content of each tube was split, and one-half of the material was processed for RNA isolation immediately; the other half was stored at -80°C. Total RNA was then isolated using the standard RNA Blood protocol on the ABI PRISM™ 6100 Nucleic Acid PrepStation in 96-well format (ABI). RNA was eluted in the ABI elution buffer and stored at -80°C and quantified by measuring optical density reading at 260 nm. Sample quality was assessed by using an Agilent 2100 Bioanalyzer (Agilent Technologies, Palo Alto, CA, USA).

For PBMC isolation, 3–5 ml blood were collected into heparinized tubes (BD, San Jose, CA, USA). Blood was spun at 1500*g* for 20 min to remove plasma. The blood cell pellet was resuspended in an equal amount of HBSS (Gibco BRL/Invitrogen,

Carlsbad, CA, USA). Lympholyte-Rat (Cedarlane Labs, Hornby, Ontario, Canada) (5 ml) was added to a 15-ml flacon tube. The cell suspension was then carefully layered over the top of the Lympholyte-Rat. The tube was centrifuged for 40 min at 1500*g*. The white cell layer was collected using a Pasteur pipette. PBMC palettes were rinsed twice with cold HBSS and stored in RNALater (Ambion, Austin, TX, USA) until RNA isolation. RNA purification was performed using RiboPure™-Blood (Ambion) according to the manufacturer's instructions.

Affymetrix microarray and gene ontology analyses

A total of 2 μg total RNA along with 5 μg glycogen (Ambion) carrier were precipitated for sample labelling in order to remove the EDTA in the RNA elution buffer, which might interfere enzymatically with the labelling reaction. RNA labelling was processed according to the standard Affymetrix protocol (Joyce et al. 2001, Shou et al. 2002). Briefly, RNAs were reverse transcribed and double-stranded cDNAs were synthesized and purified using the Affymetrix microarray-cleaning module (Affymetrix). Biotin-labelled cRNAs were synthesized manually using the BioArray™ HighYield™ RNA Transcript Labeling Kit (Enzo Life Science, Inc., Farmingdale, NY, USA). The labelled cRNA was purified, fragmented and hybridized to human U133 plus 2 or rat RAE 230A chips (Affymetrix). Signal intensities and detection calls of probesets from each hybridized genechip were extracted using Affymetrix's microarray suite 5.1 (MAS 5.1) adopting default parameters.

For pathway analysis, the 5804 rat and 18 024 human probesets that were called 'expressed' by all four chips were mapped to LocusLink and annotated using various pathway information from the Lilly in-house annotation database, Swiss-Prot, Transpath and KEGG. Biological process classification was performed by using integrated annotation for each gene. The number of genes that fell into each biological process category was calculated and presented as the percentage over the total number of the genes.

Chip signal correlation analysis

Chip signals were normalized using all probesets to reach the overall 2%-trimmed mean of 1500 for each chip. The human HG-U133_plus_2 and rat RAE230A chips have 54 675 and 15 923 probesets, respectively. To reduce the noise caused by probesets expressed at very low level, only probesets that called 'present' (i.e. expressed) at least once by the MAS5 algorithm from the four chips were used in the calculation of chip signal correlation. This filtering resulted in 21 308 and 6061 probesets for human and rat data, respectively. A cubic root transformation was further applied to the chip signals before the estimation of correlation coefficients to reach a relatively uniform variance of gene expression across the large range of gene expression signal, i.e. let y_i^0, z_i^0 be the raw signal values for chips Y and Z, $i = 1, 2, \ldots, n$, corresponding to probesets on the chip, in our case n are 21 308 and 6061 for human and rat, respectively, then:

$$y_i = (y_i^0)^{1/3}, \quad z_i = (z_i^0)^{1/3} \qquad \text{(cubic root transformaton)},$$

and the correlation coefficient of the signals of chips Y and Z was calculated as:

$$r_{YZ} = \frac{\mathrm{cov}(y_i, z_i)}{\sqrt{S_y^2 S_z^2}},$$

where $\mathrm{cov}(y_i, z_i)$ is the covariance of y_i and z_i, and s_y^2 and s_z^2 are the variance of signals for chips Y and Z, respectively:

$$\mathrm{cov}(y_i, z_i) = \frac{1}{n-1}\sum_{i=1}^{n}(y_i - y_{mean})(z_i - z_{mean}),$$

$$s_y^2 = \frac{1}{n-1}\sum_{i=1}^{n}(y_i - y_{mean})^2, \quad s_z^2 = \frac{1}{n-1}\sum_{i=1}^{n}(z_i - z_{mean})^2.$$

Results

Yield and quality assessment of total blood RNA isolated using a TEMPUS tube

To assess a reliable method to isolate total RNA from whole blood for microarray applications, we tested Applied BioSystems' blood collection and RNA extraction platform. We obtained a satisfactory RNA yield from whole blood with the yield from rat blood being ten-fold higher than that of human (23.7 μg ml^{-1} whole blood (rat) versus 2.7 μg ml^{-1} whole blood (human)). We further assessed the quality of RNA isolated using the current approach by analysing RNA samples on Agilent's 2100 Bioanalyzer. The electronic gel view and electropherogram of four rat RNA samples are shown in Figure 1A, C. RNA appears to be intact with no sign of degradation and no apparent genomic DNA contamination. The human blood RNA samples also appear to be of high quality (data not shown). The results suggest that the current method could provide sufficient and good quality RNA for Affymetrix GeneChip studies.

Sample labelling and chip performance

We examined if the RNA isolated from the whole blood was suitable for labelling and Affymetrix microarray analyses. Starting with 2 μg total RNA, every sample was successfully labelled. The labelled cRNA yield from *in vitro* transcription (IVT) is shown in Table I. IVT yield from 2 μg input RNA is sufficient for multiple rounds of chip hybridization, since only 15 μg IVT are needed for one round of chip hybridization. We then fragmented the IVT and hybridized them to human U133 Plus2 and rat RAE230A chips for the human and rat blood samples, respectively. The quality of the raw chip data was first assessed by examining the standard quality control parameters shown in Table II. The background was low; and scaling factors were at around 10 (chips were normalized to a target intensity of 1500). The actin 3′:5′ signal ratio was close to 1, which suggests no RNA degradation. Most importantly, we obtained an average of approximately 40% of the total probesets that were called expressed by the MAS5 algorithm. The current observed sensitivity was in the range for purified PBMCs (around 40–45%), but it was higher than the PAXgene whole blood RNA isolation methods (20–30%) (Feezor et al. 2004). As a bench marker, we observed that around 20% of the total probesets were called expressed when using the PAXgene approach in an independent experiment. Our data

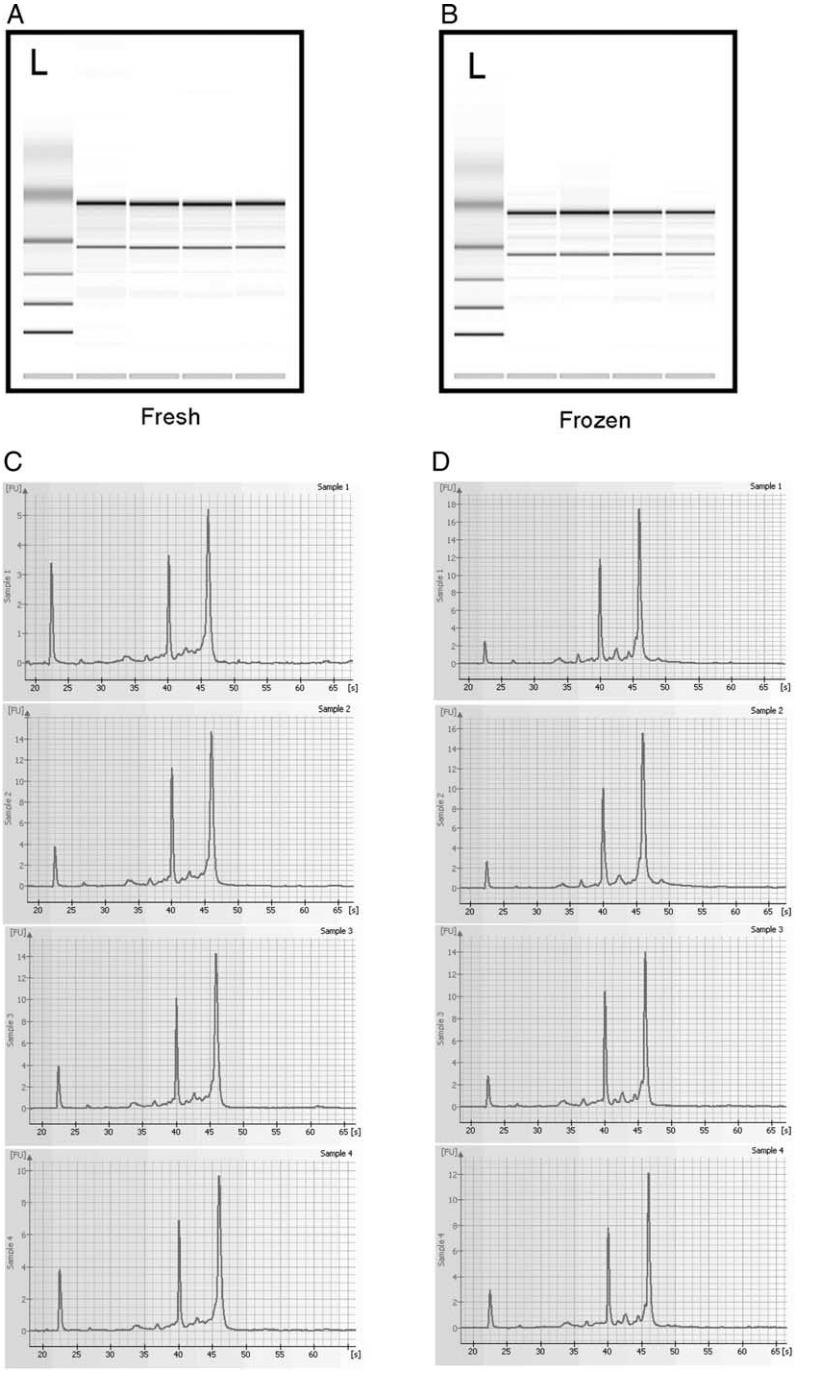

Figure 1. RNA quality examination on an Agilent 2100 Bioanalyzer. A total of 1 μl undiluted RNA was examined for RNA quality using the bioanalyser. An electronic gel view for four sampler rat RNA samples is shown. Note the sharp bands for 18s and 28s rRNAs, respectively. There is no sign of RNA degradation or genomic DNA contamination. (A) Fresh isolated RNA samples; and (B) frozen samples. The electropherogram of the fresh and frozen samples are shown in (C) and (D), respectively. L, RNA 6000 ladder (Ambion, Austin, TX, USA).

Table I. Labelled cRNA (IVT) yield from 2 μg input whole blood total RNA.

Sample ID	IVT yield (μg)
Human sample 1, fresh	33.1
Human sample 2, fresh	34.6
Human sample 1, frozen	46.1
Human sample 2, frozen	41.8
Rat sample 1, fresh	51.8
Rat sample 2, fresh	63.4
Rat sample 1, frozen	79.2
Rat sample 2, frozen	69.1

Sample ID, rat or human whole blood RNA sample identification; IVT yield, labelled cRNA yield from 2 μg input total RNA. RNA was reverse transcribed and double cDNA was used as the template for *in vitro* transcription in which biotin-conjugated ribonucleotides were incorporated. Note that labelled cRNA yield is sufficient for multiple chip hybridization. IVT, *in vitro* transcription.

indicated an improvement in whole blood RNA isolation and labelling, in terms of preserving the quantitative representation of messenger RNA.

We performed gene ontology analysis using the probesets that were called present in all the chips for a given species (5804 for rat and 18 024 for human). The probesets were mapped to LocusLink and then to biological pathways using biological process classification. We observed similar coverage between the human and rat samples. The ten most representative pathways are shown in Figure 2. The key pathways listed as the top categories are cell signalling, immune response, etc. The rat and human orthologous genes were further retrieved from NCBI HomoloGene (Wheeler et al. 2003). The number of orthologues in each category is shown in parenthesis (Figure 2).

Assessment of haemoglobin gene expression across different blood RNA isolation methods

One challenge to the profiling of whole blood is the presence of highly abundant haemoglobin messenger in the total RNA, as well as other ions and protein contamination, all of which could negatively impact whole genome expression analyses (Fan & Hegde 2005). We thus examined the haemoglobin expression in

Table II. Selected quality control parameters of chip hybridization.

Sample ID	Chip	Scaling factor	Bkgd	P+M (%)	Actin 3′:5′
Human sample 1, fresh	U133, plus2	9.2	57.1	38.9	1.17
Human sample 2, fresh	U133, plus2	8.7	60.3	38.8	1.21
Human sample 1, frozen	U133, plus2	9.5	56.0	40.6	1.22
Human sample 2, frozen	U133, plus2	10.1	55.8	39.0	1.12
Rat sample 1, fresh	RAE230A	13.1	62.4	37.7	1.36
Rat sample 2, fresh	RAE230A	13.1	54.4	39.1	1.07
Rat sample 1, frozen	RAE230A	12.9	54.2	39.6	1.2
Rat sample 2, frozen	RAE230A	15.0	53.7	38.6	1.21

Sample ID, rat or human whole blood RNA sample identification; chip, the types of chip used for the rat and human samples, respectively; scaling factor, the scaling factor used for whole chip normalization to the target intensity of 1500 in MAS5; Bkgd, background level reported for a given chip; P+M (%), percentage of the probesets called present or marginal present (i.e. percentage of the probesets called expressed); actin 3′:5′, the 3′:5′ ratio for actin.

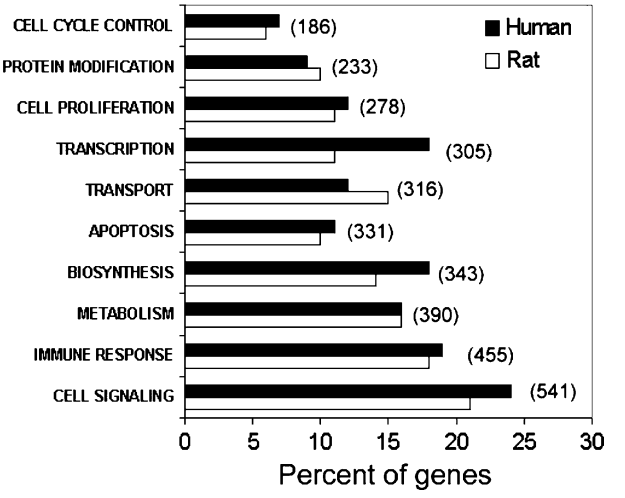

Figure 2. Gene ontology revealed by whole blood transcript profiling. The 5208 (rat) and 18 024 (human) present probesets were mapped to the LocusLink and then to biological pathways. The percentages of the genes fallen into each category over the total number of genes with known pathway annotation were plotted for the ten most representative pathways. Numbers shown in parentheses are the numbers of the orthologues between rat and human in each category.

our samples. Haemoglobin genes appeared to be the most highly expressed genes, especially the beta and alpha subunits. The level of haemoglobin expression is approximately 15-fold higher than the mean signal value in the human samples, while around 30 in the rat samples. We compared the expression of haemoglobin genes in the RNA samples prepared using different methods. Figure 3 shows the heat map visualization of the normalized expression. As expected, haemoglobin expression in the PBMC samples is the lowest. The current method using the TEMPUS tubes resulted in an about a 50% increase in relative haemoglobin gene abundance in comparison with the PBMC samples. However, we observed a four-fold reduction in haemoglobin expression as compared with the RNA isolated using PAXgene whole blood RNA isolation approach.

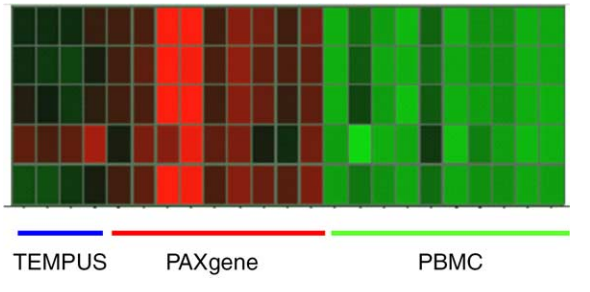

Figure 3. Visualization of relative haemoglobin gene expression in rat blood RNA samples isolated using different methods. The signal values of five rat haemoglobin probe sets were Z-score normalized and plotted across samples prepared using different methods. Note that the PBMC method has the lowest haemoglobin expression level, while the PAXgene whole blood isolation method has the highest haemoglobin expression. PBMC, peripheral blood mononuclear isolated using the Ficoll gradient; PAXgene, one commonly used whole blood RNA isolation approach; TEMPUS, present method using the ABI TEMPUS platform.

Effects of sample freeze on RNA yield and chip performance

One of the challenges with profiling blood for biomarker discovery is sample handling when multiple sites are involved and immediate RNA isolation is not ideal. Freezing down cell lysates and performing RNA isolation later in a centralized laboratory provides a possible solution to overcome this challenge. We thus assessed the effects of sample freeze–thaw on RNA yield, quality and chip performance. Figure 4 shows that no reduction in RNA yield was observed following one cycle of freeze–thaw. In addition, the integrity of the RNA was still of high quality when viewed on the Agilent 2100 Bioanalyzer (Figure 1B). There was no difference in IVT yield or in overall chip performance (Tables I and II). To address further the effects of sample freeze on chip performance, we analysed the data statistically by calculating the correlation coefficients among individual chips. It is well known that in the Affymetrix microarray platform the variance of gene expression increases with signal intensity on a raw scale and it decreases with signal intensity on a log scale (Huang et al. 2003). A cubic root transformation was applied to the chip signals before the estimation of correlation coefficients. We observed a tight correlation coefficient between the frozen and matched fresh sample of both species (Table III). These data suggest that sample freeze has minimal effects on global gene expression in whole blood RNA isolated using the current approach.

Discussion

To establish a reliable method allowing us to isolate high-quality RNA from whole blood for microarray studies, we evaluated Applied BioSystems' blood collection and RNA extraction platform. It was demonstrated that the RNA isolated from this system was of high quality and suitable for Affymetrix microarray applications. An adequate amount of RNA was obtained from both human and rat whole blood samples. The RNA showed a reduction in haemoglobin expression resulting in an increase in the number of probesets expressed when compared with previous array data using other whole blood isolation approaches. Furthermore, sample freeze before isolation has

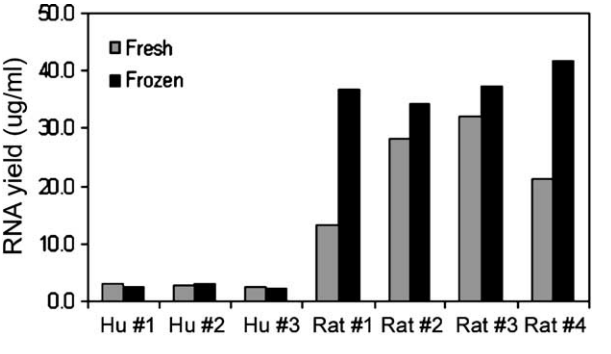

Figure 4. Effects of sample freeze on RNA yield from whole blood preparation. Whole blood lysate were equally split. Half the samples were used for RNA isolation immediately after blood collection and lysis; the rest were frozen at $-80^{\circ}C$ for 3 weeks. The frozen samples were thawed on ice and the RNA isolated on an ABI PRISM^{TM} 6100 Nucleic Acid PrepStation. The RNA yield was determined by measuring the optical density at 260 nm. Grey bars, fresh samples; black bars, frozen samples. Note that there is no substantial reduction in RNA yields by sample freeze.

Table III. Summary of correlation coefficient analysis of fresh and frozen human and rat samples.

	Human 1, fresh	Human 2, fresh	Human 1, frozen	Human 2, frozen
Human samples, 21 308 probesets:				
Human 1, fresh	1	0.988	0.985	0.984
Human 2, fresh	0.988	1	0.987	0.988
Human 1, frozen	0.985	0.987	1	0.987
Human 2, frozen	0.984	0.988	0.987	1
	Rat 1, fresh	Rat 2, fresh	Rat 1, frozen	Rat 2, frozen
Rat samples, 6061 probesets:				
Rat 1, fresh	1	0.988	0.989	0.984
Rat 2, fresh	0.988	1	0.988	0.99
Rat 1, frozen	0.989	0.988	1	0.989
Rat 2, frozen	0.984	0.99	0.989	1

Correlation coefficient was calculated as described in the Materials and methods. Probesets that were expressed at least once were subjected to analysis. Cubic root transformation was applied to stabilize the variance over the probesets before the correlation coefficients were calculated. Note that there is no major impact on global gene expression by sample freeze and thaw.

minimal effects on global gene expression when this approach is used. The present platform proved to be a rapid and reliable source for blood profiling in support of blood cell genomics.

The standard Affymetrix RNA-labelling protocol for gene array studies requires 5 μg input RNA. Obtaining a large amount of blood for RNA isolation is challenging and various methods have been developed to label smaller quantities of RNA (McClintick et al. 2003, Zhu et al. 2003, Glanzer & Eberwine 2004, Kenzelmann et al. 2004, Marciano et al. 2004, Wilson et al. 2004, Shou et al. 2005). In the current study, although only 2 μg instead of 5 μg input RNA were labelled, we obtained a successful sample labelling to produce good chip performance. The current procedure is likely to be extendable to most instances of blood cell transcript profiling. Nevertheless, if only a subpopulation of blood cells needs to be studied, obtaining enough RNA from them will then be very restricted. Under such a circumstance, it would be useful to optimize further the system to allow smaller blood sample profiling.

There are two major blood cell gene profiling platforms currently available: the whole blood cell and PBMC approaches (Fan & Hegde 2005). The PBMC method focuses on peripheral lymphocytes and is a powerful blood cell profiling strategy to assess immune responses from lymphocytes (DePrimo et al. 2003, Jison et al. 2004, Rus et al. 2004). However, isolation of PBMC could be time consuming and labour intensive, and may also involve undesired *ex vivo* activation of lymphocytes during cell fractionation. Moreover, for applications in which there is a broader range of cell populations of interest, such as erythrocytes and granulocytes, the whole blood isolation approach is more appropriate. One important finding of the present study is the improvement in reducing haemoglobin interference with sample labelling and chip performance. Haemoglobin interference has been one of the challenges that limits the use of whole blood gene expression profiling (Fan & Hegde 2005). A few other remedial approaches have been attempted such as selective erythrocyte lysis and the reverse selection approach by using the haemoglobin-specific oligos to deplete haemoglobin messengers. However, these approaches are less robust, highly variable and less ideal for clinical applications (e.g. haemoglobin removal sometimes might not

be sufficient or the depletion procedure requires multiple step sample handling including enzymatic treatment that is difficult to control). In this light, we examined the haemoglobin effects in the present study and found minimal haemoglobin interference. It was observed that the residual haemoglobin expression in the samples prepared using the current method was in the effective range of those achieved by using purified PBMC. We revealed the expression of around 40% of the genome, recapitulating the major biological pathways by the gene ontology analysis. Nevertheless, with around 40% of the probesets being called expressed, subtle changes can be identified if the experiment is properly designed (Kaiser et al. 2004). The slightly lower than PBMC number of probesets called expressed could be attributable to the residual expression of haemoglobin, or alternatively to the loss of other transcripts that were non-selectively removed along with the haemoglobin during the RNA isolation. It is challenging to distinguish two such possibilities. However, one needs to be aware of the possible bias introduced by the RNA isolation method when interpreting blood cell transcript profiling studies. It was also demonstrated that sample freeze has nearly no negative effects on RNA yield, quality or chip performance, which make it an ideal choice for clinical applications. Indeed, we observed an increased RNA yield from the frozen samples. The exact mechanism for this is not fully understood. It is speculated that the freeze–thawing prolonged incubation facilitated the lysis of cells and thus improved the yield. It could also be due to well-by-well variability. Sample stability at standard storage conditions at room temperature is warranted for future studies.

In summary, the high RNA yield, quality, lysate stability, ease of sample handling and feasibility for high-throughput laboratory automation make the current approach attractive for clinical implementation for genomic target/biomarker discovery.

Acknowledgements

The authors are grateful to Jeff Fill and Mark Willey for help with blood collection. They thank Mark Rekhter, Peter Mitchell and members of the functional genomics group for helpful discussions. They also thank John Calley for gene annotation as well as IT support from the bioinformatics/IT group. J. S. and C. D. contributed equally to this study.

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