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Comparison of Amplification Methods to Produce Affymetrix GeneChip® Target Material

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16. Abstract Whole blood from living subjects is a convenient matrix to use as a source of RNA for microarray experiments with human subjects especially when subject material is collected at a location other than the collaborating site conducting the microarray work. Collection methods for whole blood that include stabilization of the RNA are known but suffer from issues of decreased sensitivity due to the large amount of globin RNA present from reticulocyte lysis. The experiments presented here were designed to test a globin-RNA reduction protocol in conjunction with three different amplification methods. Statistical analysis of the six different protocols, coupled with post-hybridization quality assurance methods, revealed that an amplification protocol that yielded a fragmented biotin-labeled cDNA product resulted in the highest Percent Present calls from the Affymetrix analysis software and the least methodology based variability. Based on these results, this amplification protocol is expected to lead to the greatest sensitivity and accuracy for differential expression testing of the six amplification methods tested.			
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COMPARISON OF AMPLIFICATION METHODS TO PRODUCE AFFYMETRIX GENECHIP® TARGET MATERIAL

INTRODUCTION

Minimally invasive, simple sample collection is a distinct advantage when conducting experiments using completely healthy subjects. Whole blood from live subjects is a convenient source of material for microarray experiments performed with human subjects. Furthermore, gene regulation in blood has been shown to be reactive to an array of conditions (1-5). However, globin-RNA is a known interfering factor in microarray experiments performed using whole blood total RNA as starting material (6). The object of this work is to determine the qualitative and quantitative differences of target materials prepared from whole blood total RNA treated or not treated with a globin-RNA reduction method for hybridization to Affymetrix GeneChips® (Affymetrix, Santa Clara, CA). Three methods were compared: the Eberwine IVT procedure as a one-cycle protocol (sold in kit form by Affymetrix and others), the Eberwine IVT procedure modified to be a two-cycle protocol (used in this study in kit form from Arcturus, Mountain View, CA), and the Ovation biotin amplification kit by NuGEN Technologies (San Carlos, CA).

These three methodologies differ in the required amount of input RNA, the product type, and the expected product yields. The one-cycle IVT method requires 1 to 10 µg starting material, but the other two methods can start with as little as 10 ng. Yields from the two IVT methods are 30 to 50 µg of amplified RNA whereas the Ovation method yields 7 to 10 µg of cDNA product. In contrast to the 15 µg of IVT target material needed for hybridization to hGU133plus2.0 chips, the cDNA target material needed for hybridization is 2.5 µg.

MATERIALS AND METHODS

Whole blood samples were drawn from volunteer FAA employees with informed consent. Samples were harvested and processed in two groups. Samples 34431, 34908, and 41743 were one group; samples 22162, 91946, and 95950 a second group. Blood draws were gathered into PAXgene blood tubes (Cat # 762115; Pre-Analytix, Hembrickton, GBh). Total RNA was purified from the 5 mL whole blood samples using the PAXgene RNA purification system (Cat # 762164; Qiagen) and

stored at -80°C. Quality of the RNA was assessed on RNA 6000 Nano microfluidics chips (Part # 5065-4476; Agilent Technologies, Waldbronn, Germany) under the total RNA protocol on an Agilent 2100 BioAnalyzer. A 4 µg aliquot of purified RNA from each subject was globin-RNA reduced using an RNaseH based procedure (7). This procedure binds anti-sense oligos against the 3' ends of α 1-, α 2- and β -globin transcripts to specifically target those transcripts for degradation by RNaseH after annealing.

Aliquots of globin-RNA reduced (GR) and non-globin-RNA reduced (NGR) from each subject were amplified using three different amplification methods. Two and one half micrograms of RNA from each subject was used as input material for amplification with the Affymetrix One-cycle kits (P/N 900431; Affymetrix Inc., Santa Clara, CA), according to the manufacturer's instructions. Fifty nanograms of total RNA from NGR or GR samples was used as starting material for amplification with both the RiboAmp™ OA kit (PN 12302-03; Arcturus Bioscience Inc., Mountain View, CA) and the Ovation™ biotin kit (Cat # 2300; NuGEN Technologies, Inc., San Carlos, CA), according to the respective manufacturer's instructions.

Unfragmented and fragmented reaction product was assayed on RNA 6000 Nano microfluidics chips run under the mRNA protocol. Quantification of reaction product was performed on a NanoDrop spectrophotometer (Nanodrop Technologies, Wilmington, DE). Fragmented samples were hybridized to HG-U133 Plus 2.0 GeneChips®, according to the instructions of the respective amplification kit manufacturers, depending upon the sample being hybridized.

Data analysis was performed with MAS 5.0 (Affymetrix) and the tools available in S+Array Analyzer (Insightful Corporation; Seattle, WA). A one-tailed Wilcoxon Rank-sum test was used to test the hypothesis that globin-RNA reduction and/or any other amplification protocol would increase the Percent Present calls over the non-globin-reduced samples amplified with the Affymetrix One-cycle kit. A two-tailed test was used to compare Ovation amplified samples with and without globin-RNA reduction.

RESULTS

Amplification From Total RNA

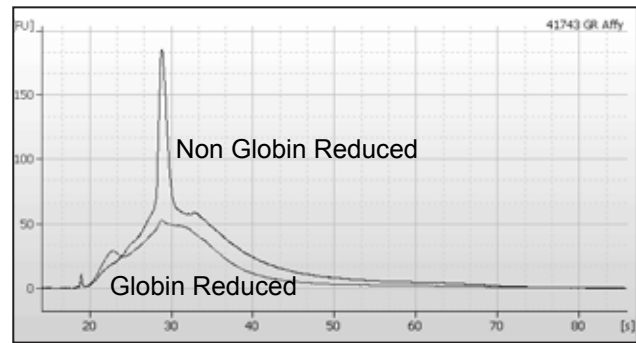
Quality assurance of sample integrity was assessed by electrophoresis in RNA Nano microfluidics chips on a BioAnalyzer 2100 using the total RNA run-protocol. RNA Integrity Numbers were calculated by the software and ranged from 8.5 to 9.2, indicating that purified RNA was of a suitable quality for microarray analysis. Next, aliquots of total RNA were globin-reduced (see Materials and Methods) and appropriate quantities of globin-reduced (GR) and non-globin-reduced (NGR) RNA amplified by one of the three methods being compared: Affymetrix One-Cycle kit (Af), Ovation™ biotin kit (Ov), or RiboAmp™ OA (Arc). Amplified material was assessed for product length and peak shape(s) with the BioAnalyzer mRNA run-protocol. Figure 1A shows an overlay of AfGR and AfNGR samples from subject 41743. Globin-RNA reduction clearly altered the product length distribution. The poly-T primed reverse-transcriptase reaction appeared to yield a large amount of full-length globin-RNA that resulted in a large spike in the IVT amplified material. That spike was markedly reduced after globin-RNA reduction.

Amplification with the Ovation™ kit is not IVT-based, rather it uses a proprietary priming system and a DNA polymerase for an isothermal continuous amplification yielding a cDNA product. This method does not result in a specific globin peak, and product lengths are less than those from one-cycle IVT reactions; however, input amounts were 50-fold less than that required for the one-cycle IVT method. There was a slight change in peak shape after globin-RNA reduction from samples amplified with the Ovation system.

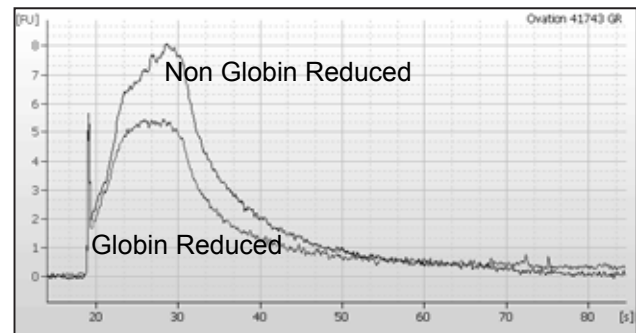
The RiboAmp™ protocol, although based on the same IVT reaction as the Eberwine procedure commercialized in the Affymetrix kit, includes a random-primed step to make input material for the second round of IVT amplification. The peak shapes from RiboAmp™-amplified material are changed by globin-RNA reduction. As expected, product lengths were much shorter with this procedure when compared with the one-cycle IVT method, likely due to the random priming step (Fig. 1C). As with the Ovation™ method, an advantage of using the RiboAmp™ system is the much lower requirement for input total RNA.

Quality Control Testing

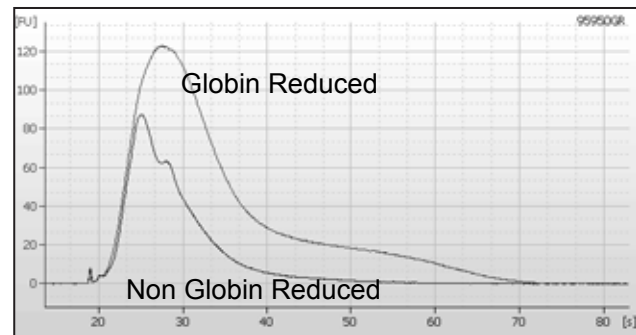
The BioAnalyzer overlays of the electropherograms from AffyNGR and AffyGR samples graphically illustrate the disappearance of the globin spike from the one-cycle amplified material, qualitatively demonstrating that the globin reduction protocol was effective in reducing the



A. Sample 41743 after amplification by the one-cycle IVT method.



B. Sample 41743 after amplification with Ovation-biotin kit.



C. Sample 95950 after amplification with the two-cycle IVT method.

Figure 1. BioAnalyzer electropherograms of representative samples after amplification by each method with and without globin-RNA reduction.

amount of globin-RNA available for IVT amplification (Fig. 1A). The electropherogram shapes between GR- and NGR-treated, Ovation-amplified samples are very similar (Fig. 1B), although a subtle change in peak shape again indicates the effectiveness of the globin-RNA reduction protocol (Fig. 1B). Similar to the Affymetrix amplified material, the RiboAmp™-amplified material without globin-RNA reduction includes a peak that can be attributed to globin-RNA that is not present in the GR samples (Fig. 1C). Product lengths of the Ovation™ and RiboAmp™ amplified samples are very similar but, as expected, the yields from the Ovation™ amplification are much lower.

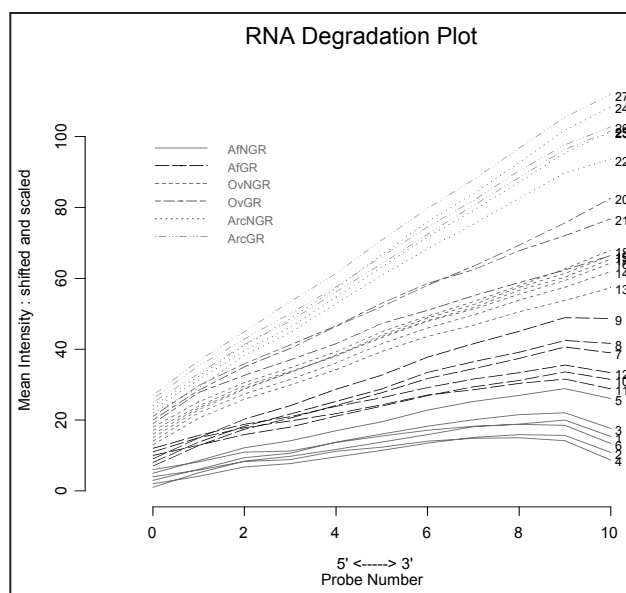


Figure 2. RNA degradation plot of amplified samples after hybridization to HG-U133 Plus 2.0 GeneChips®. Plots show relative signal intensities across probe sets from the 5' to 3' end of all probe sets on each chip. Plot slopes are informative for 3' bias of each preparation method. Plots are shifted and scaled to minimize overlap. 1-6, AffyNGR; 7-12, AffyGR; 13-18, OvNGR; 19-21, OvGR; 22-24, ArcNGR; 25-27, ArcGR.

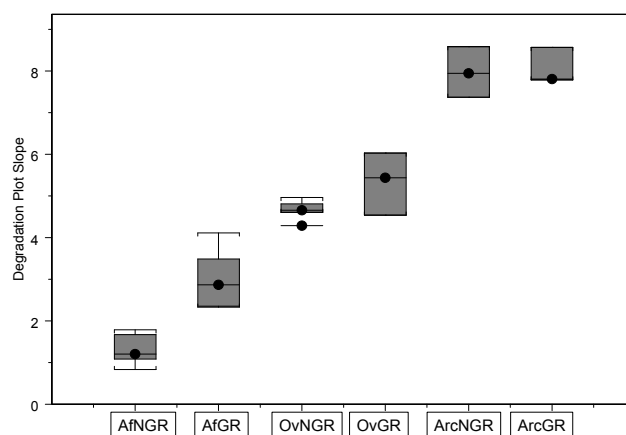


Figure 3. Median centered box-whisker plot of RNA degradation plot slopes for each treatment method. For each treatment method, RNA degradation plot slopes were plotted as median centered quartile box-whisker plots. Heights of boxes indicate 25th-to-75th quartile ranges. Since OvGR, ArcNGR and ArcGR treatment groups contain three chips, dots are median slopes and boxes indicate total range of data set.

RNA degradation plots were generated in S+ ArrayAnalyzer™ from the CEL data (Fig. 2). The test determines 3' bias of the target material across the chip by plotting signal strength within probe sets as a function of the numerical position of the probe in the probe set. In normal use, this test is used to determine the degree of degradation in the original RNA, but, in this analysis, it was used as a measure

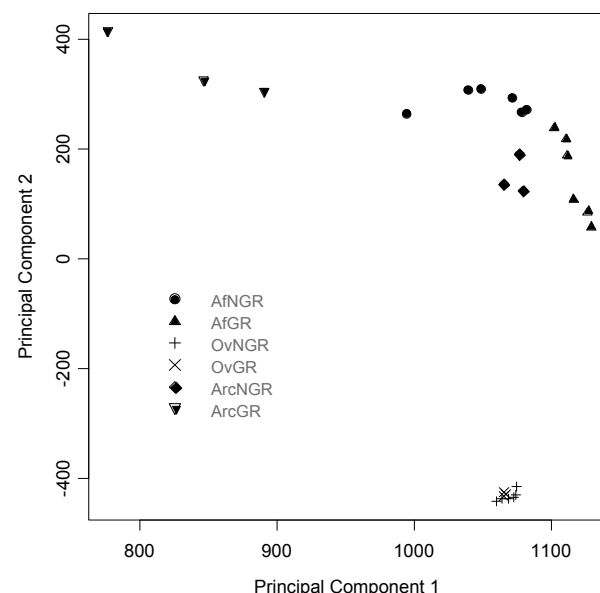


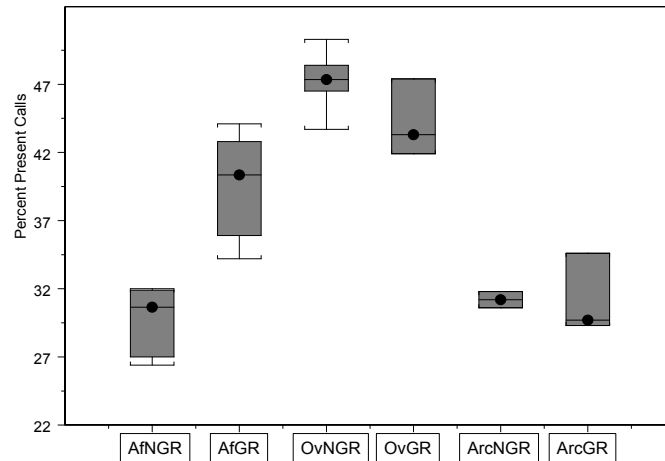
Figure 4. Principal components plot in two dimensions. Treatment groups are shown to cluster in similar regions of the plot. Similarities or differences introduced by treatment are reflected in distance of treatment groups from each other.

of change introduced into the samples as a result of treatment method. The intent was to determine the method that resulted in the least increase in 3' bias. The least 3' bias was seen in the Affymetrix NGR samples. A modest increase in 3'/5' signal ratios was seen in the Affymetrix GR samples, possibly due to a low level of non-specific hybridization by the globin oligos and subsequent cleavage by RNaseH. The Ovation™ amplified samples showed an additional increase in 3' bias over the Affymetrix samples. The greatest increase in 3'/5' signal ratios was seen in the RiboAmp™-amplified samples. Globin reduction had little effect on 3'/5' signal ratios when RNA was amplified with either the Ovation™ or RiboAmp™ kits (Fig. 2), as seen in the similarity of the slopes on the RNA degradation plot. The slopes from the degradation plots were plotted as median centered box plots (Fig. 3) and showed that samples amplified with the Ovation™ kit and without globin-RNA reduction had a much lower variability between chips. Decreased variability between chips would be expected to result in less noise in differential expression testing, thereby increasing sensitivity and accuracy.

A Principal Components plot of summarized and normalized data shows that globin reduction alters the composition of material after Affymetrix or RiboAmp™ amplification (Fig. 4). However, globin reduction has a negligible effect on RNA amplified with the Ovation™ kit, as seen by the tight grouping of all nine Ovation™ samples—irrespective of globin-RNA treatment. A marked decrease in Ovation™ amplified target material heterogeneity is evident in this assay as well.

Table 1. Percent Present Calls from MAS 5.0 Data

Sample #	AfNGR	AfGR	OvNGR	OvGR	ArcNGR	ArcGR
34413	26.4	44.1	47.9	47.4		
34908	30.9	40.5	50.3	41.9		
41743	30.4	42.8	48.4	43.3		
95950	31.9	40.2	46.5		31.8	34.6
91946	32	34.2	46.8		31.2	29.7
22162	27	35.9	43.7		30.6	29.3

**Figure 5.** Median centered box-whisker plot of Percent Present calls grouped by treatment. Heights of boxes indicate 25th-to-75th quartile ranges. Because OvGR, ArcNGR and ArcGR treatment groups contain three chips, dots are median %P and boxes indicate total range of data set.

Microarray Analysis

A summarization of the MAS 5.0 data is in Table 1; a box-whisker plot of the data is in Figure 5. Wilcoxon Rank Sum tests were performed comparing all treatments to Affymetrix amplified samples without globin-RNA reduction (AfNGR). Globin-RNA reduction clearly increased the Percent Present (%P) when material was amplified with the Affymetrix One-step kit (AfGR; $p=0.0156$; Fig. 5). The Ovation™ kit has a clear advantage over the AffyNGR method with (OvGR) or without (OvNGR) globin-RNA reduction, although statistical significance was reached only in the OvNGR samples, due to sample size (OvNGR, $p=0.0156$; OvGR, $p=.125$). Nonetheless, the trend is clear; the Ovation kit markedly increases the sensitivity of the GeneChip® assay. The increase in %P was reflected in a decrease in percent absent probe sets; percent marginal calls were unaffected. Surprisingly, the Arcturus RiboAmp™ OA kit did not increase the %P irrespective of globin-RNA treatment (Table 1; Fig. 5).

DISCUSSION

Globin-RNA decreases sensitivity and accuracy of microarray experiments performed using whole blood from living subjects as starting material. However, due to the convenience and ease-of-use of whole blood samples as starting material for microarray experiments, especially when samples are collected remotely and shipped to the site of analysis, methods are needed to improve the efficacy of whole blood as a starting material. Treatments to remove globin-RNA after RNA processing are known but necessitate additional handling, have the potential to introduce bias, and can be expensive. As shown here, amplification methods for target material preparation introduce their own bias to target material preparation, and material prepared with different methods should not be included in the same analysis or considered comparable.

To test the effects of different sample preparation methods on microarray target material preparation,

identical samples of whole blood RNA were treated using one of six different protocols. The Affymetrix One-cycle amplification kit was used as the baseline treatment in this work. To this method, the Ovation™ biotin kit from NuGEN Technologies and the RiboAmp™OA kit from Arcturus were compared. A distinct advantage of the other two amplification methods used was the requirement for at least 50-fold less material. While sample conservation is not necessarily a requirement for whole blood samples from humans, it may be an issue in work performed in smaller model organisms like mice. A large aliquot of material was globin-reduced using an RNaseH-based protocol developed at Affymetrix. This sample was further split and amplified using one of the three amplification protocols. For three of the treatment groups, data was gathered from subject samples harvested and processed on two different days. While some batch effect was evident, it was minimal and did not affect the conclusions drawn here (data not shown).

Amplified target material before fragmentation was assessed on a BioAnalyzer for relative peak shapes. In all three amplification methods, globin reduction clearly and reproducibly had an affect on peak shape when GR and NGR samples were compared. This was most dramatic for the Affymetrix-amplified material where a large, characteristic peak in NGR samples is decreased or disappears altogether. The observation that the presence or absence of the globin-RNA peak in these samples is variable (data not shown) illustrates the disadvantage of this globin-RNA reduction method in that it introduces an obvious variability to the samples. This increase in sample-to-sample variability is evident in the increased variance of the %P values after hybridization.

Quality control metrics were used to determine what changes in data quality could be expected by the respective treatments. RNA degradation plots indicated that the AffyGR, OvNGR, and OvGR samples were the most similar to the AffyNGR samples in terms of 3' bias. Hence, they would be expected to include good signal intensities across probe sets and maintain good sensitivity. Median-centered box plots of the slopes of the

degradation plots revealed that the OvNGR treatment decreased the chip-to-chip variability, compared with all other treatments. This suggests that there will be less noise, improving both sensitivity and accuracy of differential expression analysis when samples are treated with this amplification method. Principal components plots also indicated that the AffyGR- and Ovation™-amplified samples were most similar to the AffyNGR sample treatment and showed a much tighter grouping of samples amplified with the Ovation kit.

Percent Present calls were gathered from the MAS5.0-analyzed data within GCOS and analyzed. Median-centered box plots revealed that, although more variable than the NGR samples, AffyGR samples had a significantly increased number of probe sets called present, indicating an improvement in sensitivity. OvNGR samples had a higher %P than all methods tested. In addition, only the ArcNGR samples had less variability of %P across the test set. An unexpected observation was that GR decreased the %P calls when used in concert with the Ovation™ kit. This suggests that GR is unnecessary, saving at least a half-day of processing time and the expense of the procedure itself while improving sensitivity and, presumably, accuracy due to less chip-to-chip variability. The Arcturus treated samples showed no improvement over the AffyNGR samples in terms of %P, regardless of GR treatment. Globin-RNA reduction increased the variability of the %P calls for this amplification method.

In summary, three different commonly available amplification methods for making target material suitable for Affymetrix GeneChip® target material were compared in conjunction with a globin-RNA reduction protocol. The most efficient method found in these analyses was the Ovation™ biotin system. This system proved itself to be robust across a variety of samples, decreased data variability, and increased %P calls, suggesting that amplification of whole blood samples with this method will result in increased sensitivity and accuracy in microarray experiments. In addition, the method uses a minimal amount of starting material, making it ideal for small tissue sample sizes.

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