Evaluating the fidelity and reproducibility of several RNA amplification methods for cDNA microarray analysis

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Abstract

cDNA microarrays are widely used for profiling the differential gene expression of clinical biopsy samples and samples isolated by laser capture microdissection (LCM) or fluorescence assisted cell sorting (FACS). Since current labelling and hybridization protocols require microgram quantities of total RNA, techniques like RNA amplification must be used for biopsy, LCM, and FACS samples, which, at most, yield nanograms of total RNA.

The purpose of this study was to amplify two sources of total RNA using several amplification methods and compare the number of significantly up and down-regulated genes with that found by the non-amplified aminoallyl labelling of 10 micrograms total RNA. Amplification methods including the Ovation™ Aminoallyl RNA Amplification system (NuGEN Technologies), Amino Allyl MessageAmp™ aRNA kit (Ambion), BD Atlas™ SMART™ Fluorescent Probe Amplification Kit (BD Biosciences Clontech), and Global RT-PCR developed by Norman Iscove[1] were evaluated. While amplification fidelity and reproducibility of the method are of paramount importance, time, cost, and the amount of starting material required for amplification are other factors to consider.

An ideal amplification method would amplify RNA samples without distorting the abundance relationships of individual genes. The results show that amplification fidelity varied for each method evaluated and no one method was clearly a better choice. In general, we have found that the non-PCR based methods showed similar fidelity and reproducibility to PCR based methods.

Introduction

cDNA microarrays are commonly used to monitor the gene expression of different cells. Standard labelling protocols, like the aminoallyl (indirect) labelling protocol routinely used at the UHN Microarray Centre (UHNMAC), require 5-10 micrograms of total RNA[2]. Often, researchers are unable to isolate sufficient amounts of RNA from tissue samples and are required to use amplification techniques.

The Ovation™ Aminoallyl RNA Amplification and Labeling System (NuGEN) uses the patented Ribo-SPIA™ process to generate a single-stranded aminoallyl-labelled cDNA product that is the anti-sense of the mRNA starting material[3]. The DNA amplification process, called SPIA™ isothermal linear amplification, uses a SPIA™ DNA/RNA chimeric primer, DNA polymerase, and RNaseH in a homogeneous isothermal assay that provides efficient amplification of cDNA sequences generated from mRNA[3].

The Amino Allyl MessageAmp™ aRNA kit uses an amplification procedure which involves reverse transcription with an oligo(dT) primer bearing a T7 promoter and in vitro transcription of the cDNA with T7 RNA Polymerase to generate hundreds of anti-sense RNA copies of each mRNA in the sample. This method of amplification is commonly used because RNA polymerase activity is generally not affected by the concentration of the individual templates in a complex mixture, and, for the few templates that are transcribed more or less efficiently than other templates, the amplification bias is typically equivalent in all samples[4].

BD Super SMART™ cDNA synthesis is a novel, PCR-based method of RNA amplification that involves the SMART™ (Switching Mechanism At the 5' end of RNA Transcript) technology. During reverse transcription, when the enzyme reaches the 5' end of the mRNA, the enzyme's terminal transferase activity adds a few additional nucleotides to the 3' end of the cDNA[5]. The BD SMART II™ A oligonucleotide base-pairs with the additional nucleotides at the 3' end of the cDNA creating an extended template. The RT enzyme then switches template and continues replicating to the end of the oligonucleotide. The BD SMART™ sequence and the polyA sequence are then used as universal priming sites for cDNA amplification by PCR[5].

Iscove *et al*. have developed a rapid and highly optimized global RT-PCR procedure that can preserve abundance relationships through amplification as high as 3 x 10^{11}-fold. Iscove *et al*. acknowledge that exponential amplification is believed to degrade abundance relationships because cDNAs of differing lengths and composition would be amplified with varying efficiencies. The most important design choice in the Global RT-PCR method was to limit the extent of reverse transcription to only a few hundred bases of extreme 3' sequence by limiting deoxynucleotide concentrations and the time of the reaction[1]. These conditions were intended to provide a more uniform likelihood of sampling individual mRNA transcripts, and a more uniform amplification efficiency across all cycles[1]. While this technique may present a bias toward the amplification of the 3' end of mRNA sequences, it allows researchers to amplify RNA when only picogram quantities of total RNA are available.

There are many statistical software packages available for the analysis of microarray data. Significance Analysis of Microarrays (SAM; Stanford University) is a statistical technique for finding significant genes in a set of microarray experiments. The input to SAM is gene expression measurements (the normalized LexE/LexR ratio (log2) for each gene) from a set of microarray hybridizations and a response variable from each experiment[6]. In the one-class response variable, SAM tests whether the mean gene expression differs from zero.

Method

Total RNA from cultured HeLa cells and commercially prepared Universal Human Reference RNA (UHRR; Stratagene) were amplified using kits from Ambion, BD Biosciences Clontech, and NuGEN Technologies, and a method for Global RT-PCR described by Iscove *et al*. The labelled cDNA or aRNA was hybridized to ssHum19k7 arrays (UHN Microarray Centre; 19,008 human EST clones). Signal intensities and ratios achieved using each method were compared to that of microarrays hybridized with labelled-cDNA generated from non-amplified HeLa and UHRR total RNA.

For all hybridizations (amplified and non-amplified samples), the HeLa and UHRR samples were labelled with Cy3 and Cy5 respectively. To minimize the variation between the amplification methods, one technician performed the experiments using cDNA microarrays manufactured from the same production run, RNA was aliquoted from the same stock tube, labelling reagents were from a common pool, and a master mix was used whenever possible. Each amplification method was carried out on a different day. To account for the inherent day-to-day variation, non-amplified HeLa and UHRR samples (10 micrograms total RNA labelled following the standard aminoallyl UHN MAC labelling protocol[7]) were labelled and hybridized, in triplicate, along side the amplified samples.

Table 1 outlines the amount of RNA amplified for each of the amplification techniques and the approximate time to generate aminoallyl labelled aRNA/cDNA.

After generating labelled cDNA/aRNA following the manufacturer's protocol, the samples were hybridized to ssHum19k7 arrays following the UHN Microarray Centre's standard hybridization protocol[7]. The slides were also washed following the standard washing protocol and scanned on the same day using the ScanArray4000 (Perkin Elmer) scanner. Data was quantified using ArrayVision™ (Imaging Research) and loaded into GeneSpring™ (Silicon Genetics) for normalization (LOWESS) and visualization.

Table 1. A summary of the amount of total RNA amplified by each amplification method and the approximate time to generate aminoallyl-labelled cDNA/aRNA.

Amplification Method	*Amount of total RNA recommended for amplification*	*Amount of total RNA amplified in this study*	*Time to generate aminoallyl labelled aRNA/cDNA*
Ovation™ (NuGEN)	5-100ng	20ng and 100ng	5 hours
MessageAmp™ (Ambion)	100-2000 ng	100ng and 500ng	11-19 hours
BD Atlas™ SMART™ (BD Clontech)	20-1000ng	100ng	8 hours
Global RT-PCR (Iscove et.al.)	As little as 20pg	200pg and 20ng	10 hours

Results

In exploratory data analysis, it is advantageous to analyze the data in a number of different ways. Of the two analysis paths chosen, both used one-class SAM to determine the number of significant differentially expressed genes from the sets of arrays hybridized with non-amplified samples. However, one method considered each set of 3 non-amplified hybridizations separately and the other considered all of the non-amplified hybridizations (a total of 15 hybridizations) together.

To assess the fidelity of each amplification method, control spots and repeated sequences were removed, leaving 17,774 elements for analysis. To account for day-to-day variability, since each amplification method was evaluated on a different day, a set of 3 arrays hybridized with non-amplified "control" samples was performed. Using SAM, the one-class response analysis was performed separately on each set of non-amplified samples to find the number of significantly differentially expressed genes. One-class SAM analysis was then performed on this sub-set of filtered genes for the arrays hybridized with amplified samples on the same day. The number of positive significant genes indicates the number of genes for which LexE (HeLa) appears to be in greater abundance compared with LexR (UHRR) and the number of negative significant genes indicates the number of genes for which LexE is lower in abundance compared with LexR. Venn diagrams illustrate the overlap among each sub-set of positive and negative significant genes (Figure 1).

It is interesting to note that while the number of significant genes found in each set of arrays hybridized with non-amplified samples fluctuates, the percentage of positive and negative significant genes remains similar (Table 2), with the exception of one of the four sets. A Venn diagram (Figure 3) illustrates the overlap among the significant genes from the non-amplified samples for three of amplification methods.

Table 2. After filtering the 19,200 array elements (to remove control spots, blanks, etc), 17,774 elements remained for analysis. Each amplification method was performed on a different day. To account for day-to-day variability, a set of 3 arrays hybridized with non-amplified "control" samples was also run. Using one-class SAM analysis, the number of significantly differentially expressed genes was determined for each set of non-amplified samples. One-class SAM analysis was then performed on this sub-set of filtered genes for the amplified samples.

Sample set	Number of genes for one-class SAM analysis	Number of significant genes	Number of positive significant genes	Number of negative significant genes
Non-amplified (Ovation set)	17744	1189	127	1062
Ovation 20ng amplified	1189	498	37	461
Ovation 100ng amplified	1189	715	29	686
Non-amplified (MessageAmp set)	17744	1645	366	1279
MessageAmp 100ng amplified	1645	276	28	248
MessageAmp 500ng amplified	1645	308	58	250
Non-amplified (RT-PCR set)	17744	1912	384	1528
RT-PCR 200pg amplified	1912	688	27	661
RT-PCR 20ng amplified	1912	20	0	20
Non-amplifed (BD SMART set)	17774	3106	1301	1805
BD SMART 100ng amplified	3106	945	133	812

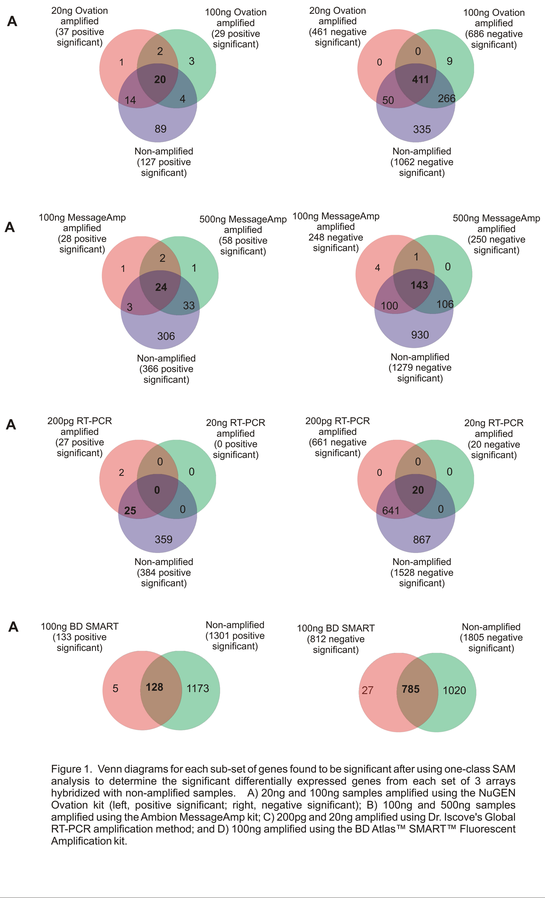

Figure 1. Venn diagrams for each sub-set of genes found to be significant after using one-class SAM analysis to determine the significant differentially expressed genes from each set of 3 arrays hybridized with non-amplified samples. A) 20ng and 100ng samples amplified using the NuGEN Ovation kit (left, positive significant; right, negative significant); B) 100ng and 500ng samples amplified using the Ambion MessageAmp kit; C) 200pg and 20ng amplified using Dr. Iscove's Global RT-PCR amplification method; and D) 100ng amplified using the BD Atlas™ SMART™ Fluorescent Amplification kit.

In the second analysis path, one-class SAM analysis was performed on all microarrays hybridized with non-amplified RNA (total of 15 hybridizations). We found that as the number of replicates analyzed by SAM increased, the number of significant genes (8933) also increased. We also determined how many of the 8933 significantly up or down regulated genes from the non-amplified set were also significantly expressed after amplification. To do this, one-class SAM was performed on this sub-set of genes (8933) for each amplification group. The overlap among significant genes from non-amplified samples and amplified samples was also determined (data not shown). ANOVA analysis was performed on the set of 8933 significant genes from the Ovation, MessageAmp, and Global RT-PCR amplified samples. The changes in gene expression across the three amplification methods can be visualized in Figure 4. After filtering 8933 significant genes, a set of the 8811 non-significant genes remained. This set represents the genes that were not significantly differentially expressed between the LexE (HeLa) and LexR (UHRR) non-amplified samples. One-class SAM was also performed on the set of 8811 non-significant genes for each amplification method, from each amount of starting material amplified. The number of significant genes found from this analysis indicates the number of genes that appear to be differentially expressed only when amplified.

Besides amplification fidelity, another important factor to consider when evaluating amplification techniques is the reproducibility of each method. Ideally, an amplification method should be reproducible as known biases of the amplification method, once identified, can be taken into account during analysis. Plots of Coefficient of Variance (CV) v intensity for each element was generated for each triplicate set of hybridizations and, as expected, the CV was greater at lower signal intensities and improved as signal intensities increased (data not shown). The reproducibility of data between replicates can also be seen in the cluster diagrams for each amplification method (Figure 5).

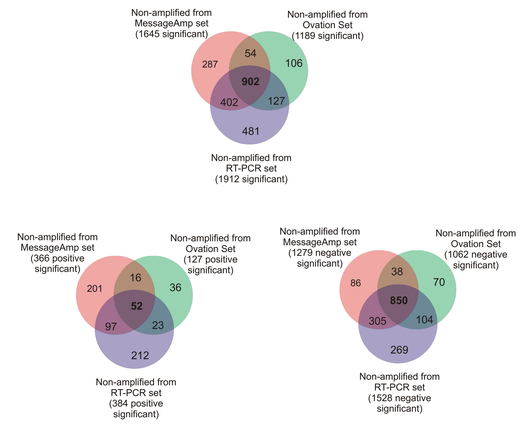

Figure 2. Venn diagrams illustrating the overlap among the significant genes (Top, all significant; bottom left, positive significant; bottom right, negative significant) found using SAM one-class response analysis on each set of 3 arrays hybridized with non-amplified samples on different days.

Discussion

Although every amplification method comes with a recommended range of starting material to amplify, the discrepancy in the number of significant genes identified by the same amplification method but from different amounts of starting material may suggest that there is an optimal amount for each method.

Comparison of the overlap among significant genes from amplified and non-amplified data indicate that some of the genes found to be significant in the non-amplified set were also significant in the amplified sets. From the set of genes identified as "non-changers" by analysis of the non-amplified samples (8811 genes), the number of significantly differentially expressed genes identified by each amplification method also varied, however, as expected, the number of significant genes was relatively low. Further validation of these genes, using techniques like quantitative PCR, would be needed to see if these genes are indeed differentially expressed between the two RNA samples.

Using the Agilent 2100 Bioanalyzer, the size of the amplified product (aminoallyl labelled cDNA or aRNA) was also determined (data not shown). Variation in cDNA/aRNA length may play an important role in hybridization efficiency. Also, a closer look at the amplification technique employed by each kit may explain some loss of fidelity. For example, T7-based amplification and Global RT-PCR may over represent the 3' end of the mRNA, especially for long transcripts. For PCR-based methods, genes containing repetitive sequences may be under (or over) represented in the amplified product.

Further analysis of the data is underway. Analysis of the actual genes found to be common among the significant genes identified by each amplification method will be performed. Also, genes that were found to be significant by one amplification method but not the others will be investigated to determine if there is a bias present that would explain the loss of amplification fidelity. A closer look at the actual signal intensities will also be done to see which of the genes, and how many, are appearing to be differentially expressed when in fact the signal intensity is just in the low (and highly variable) range.

Future experiments have also been planned. For example, we intend to amplify one RNA sample, and label with one fluor, and co-hybridize all amplified samples with a common non-amplified reference labelled with the other fluor. Upon analyses of the data from this set of hybridizations, we hope to determine whether it is best to amplify both RNA samples in an experiment or amplify the experimental sample and co-hybridize with a non-amplified reference sample.

References:

1. Iscove et al. Representation is faithfully preserved in global cDNA amplified exponentially from sub-picogram quantities of mRNA. Nature Biotechnology, 20:940-943, 2002
2. University Health Network (UHN) Microarray Centre website: http://www.microarrays.ca/support/Amino%20Allyl%20Labeling.pdf
3. Ovation® Aminoallyl RNA Amplification and Labeling System. User Guide Version 1.1.
4. Amino Allyl MessageAmp® aRNA Kit. RNA Amplification and Labelign for Array Analysis. Protocol.
5. BD Atlas® SMART® Fluorescent Probe Amplification Kit User Manual.
6. Chu et al. SAM "Significance Analysis of Microarrays" Users guide and technical document.
7. University Health Network (UHN) Microarray Centre website: http://www.microarrays.ca/support/Direct%20Labelling.pdf

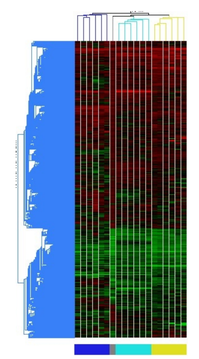

Figure 3. Hierarchical cluster of all hybridizations with samples amplified using the MessageAmp kit (dark blue), Ovation kit (light blue), and Global RT-PCR (yellow) and the non-amplified samples (averaged, grey).

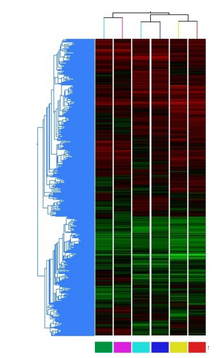

Figure 4. Hierarchical cluster of the averaged gene expression from 3 amplification methods; 100ng MessageAmp (green), 500ng MessageAmp (purple), 100ng Ovation (light blue), 20ng Ovation (dark blue), 200pg RT-PCR (yellow), and 20ng RT-PCR (red).

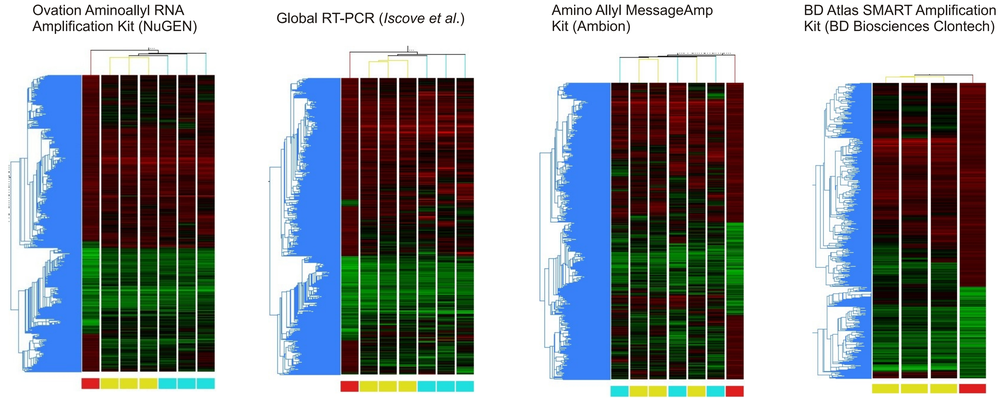

Figure 5. Hierarchical clusters for the set of 8933 significant genes for each amplification method. Left to right: Ovation Aminoallyl RNA Amplification kit (average of non-amplified samples (red) compared with triplicate hybridizations 100ng amplified (yellow) and 20ng (blue) amplified sample; Global RT-PCR (average of non-amplified samples (red) compared with triplicate hybridizations of 20ng amplified (yellow) and 200pg (blue) amplified sample; Amino Allyl MessageAmp kit (average of non-amplifed samples (red) compared with triplicate hybridizations of 100ng amplified (blue) and 500ng (yellow) amplified sample; and BD Atlas SMARTTM Fluorescent Probe Amplification Kit (average of non-amplified samples (red) compared with triplicate hybridizations of 100ng amplified (yellow) samples).

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