Research Report

RNA Amplification Results in Reproducible Microarray Data with Slight Ratio Bias

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László G. Puskás, Ágnes Zvara, László Hackler Jr., and Paul Van Hummelen¹
Hungarian Academy of Sciences, Szeged, Hungary and ¹Flanders Interuniversity Institute for Biotechnology, Leuven, Belgium

PCR results but were more sensitive in terms of detecting expressed genes. In conclusion, although amplification methods introduce slight changes in the transcript ratios compared to standard labeling, they are highly reproducible. For small sample size, in vitro transcription is the preferred method, but one should never combine different labeling strategies within a single study.

ABSTRACT

Microarray expression analysis demands large amounts of RNA that are often not available. RNA amplification techniques have been developed to overcome this problem, but limited data are available regarding the reproducibility and maintenance of original transcript ratios. We optimized and validated two amplification techniques: a modified in vitro transcription for the linear amplification of 3 µg total RNA and a SMARTTM PCR-based technique for the exponential amplification of 50 ng total RNA. To determine bias between transcript ratios, we compared the expression profiles in mouse testis versus spleen between the two amplification methods and a standard labeling protocol, using microarrays containing 4596 cDNAs spotted in duplicate. With each method, replicate hybridizations were highly reproducible. However, when comparing the amplification methods to standard labeling, correlation coefficients were lower. Twelve genes that exhibited inconsistent or contradictory expression ratios among the three methods were verified by quantitative RT-PCR. The amplification methods showed slightly more discrepancies in the expression ratios when compared to quantitative RT-

INTRODUCTION

DNA microarray technology provides a rapid and comprehensive approach to monitor simultaneously the expression levels of thousands of genes among diverse biological samples in a comparative way (5,13–15). Standard protocols of gene expression analysis require large amounts of RNA, typically 20–200 µg total RNA or 0.5–2 µg poly(A) RNA (10,17,18). To obtain these amounts of RNA, milligrams of tissue or approximately 10⁷ cells are needed. Alternatively, one can amplify the RNA. One way to amplify RNA linearly is to make use of in vitro transcription, where a T7 promoter is linked to the oligo(dT) primer and used for first-strand cDNA synthesis (17). The probe can be labeled during or after the in vitro amplification step using fluorescently labeled NTPs or dNTPs, respectively (RNA amplification protocol; <http://www.microarrays.org/pdfs/ModifiedEberwine.pdf>). Using in vitro transcription, the starting material can be limited to 100 ng to 10 µg total RNA or as little as the amount of RNA present in one cell, if two consecutive in vitro transcription cycles are performed (12). An-

other amplification strategy (RT-PCR) uses a logarithmic amplification step in which the starting material can require as little as 1 ng total RNA. This is usually accomplished by (i) ligating an anchor to the ends of the cDNA and amplifying the complex mixture using primers complementary to the anchor sequence (1,2) or (ii) using primers with 3'-end random stretches of nucleotides that can hybridize to the transcripts during reverse transcription (4,6).

One should be prudent using the amplification steps because possible bias can be introduced by these techniques. Theoretically, the risk of distorting the initial transcript level is highest for the logarithmic amplification methods. PCR-based methods are thought to be very sensitive for small changes in experimental conditions, especially for rare and long transcripts. Selective sequence amplification could also occur in the case of linear amplifications. Otherwise, in vitro transcription may also introduce bias of the initial transcript level caused by sequence-specific efficiencies of RNA polymerase, partly deriving from the uncontrolled termination of the enzyme because of a very long poly(A) tail or strong secondary structure of the message.

Although several laboratories have already used amplification steps for microarray analysis (3,7,8,11), the effect on the ratios has not been rigorously studied or published. Therefore, we present a comparative study of these amplification methods using standard labeling without amplification to investigate their reproducibility, reliability, and sensitivity.

MATERIALS AND METHODS

Construction of Microarrays

A total of 4596 PCR-amplified cDNA fragments from sequenced, verified IMAGE clones (Mouse Gem I; Incyte, USA) were spotted twice, once on each side of the slide. The cDNA inserts were amplified with M13 primers, purified with MultiScreen® PCR plate (Millipore, Brussels, Belgium), and resuspended in 20 µL 50% DMSO in an average concentration of 100 ng/µL. PCR products were arrayed on Type V silane-coated slides using a Generation III printer (both from Amersham Biosciences, Buckinghamshire, UK). The slides were blocked just before hybridization in 3.5% SSC, 0.2% SDS, and 1% BSA for 10 min at 60°C.

RNA Samples

To avoid variability caused by RNA preparation, we used highly purified commercially available poly(A) and total RNA from BD Biosciences Clontech (Erembodegem, Belgium) and Ambion (Austin, TX, USA). All experiments were performed with RNA from mouse adult spleen and testis.

Direct Labeling of RNA during Reverse Transcription

First-strand cDNA probes were generated by the reverse transcription of 2 µg poly(A) RNA using an anchored oligo(dT) (d-T₂₅-dA/C/G) primer (0.4 µM; Genset, Paris, France), 0.1 mM d(G/T/A)TPs, 0.05 mM dCTP (Amersham Biosciences), 0.05 mM Cy3-dCTP or Cy5-dCTP (Amersham Biosciences), 1× first-strand buffer, 10 mM DTT, and 200 U SUPERSCRIPT II™ (Invitrogen, Merelbeke, Belgium) in 20 µL total volume. The RNA and primers were denatured at 75°C for 5 min and cooled on ice before we added the other reaction components. After 2 h incubation at 42°C, mRNA was hydrolyzed in 250 mM NaOH for 15 min at 37°C. The sample was neutralized with 10 µL 2 M MOPS (0.6 M final concentration) and purified using QIAquick™ columns (Qiagen, Hilden, Germany).

Linear Amplification by In Vitro Transcription

Antisense RNA amplification was performed using a modified protocol of in vitro transcription available on <http://www.microarrays.org/pdfs/ModifiedEberwine.pdf> and published by Phillips et al. (12) and Van Gelder et al. (16). For the first-strand cDNA synthesis, 3 µg total RNA were mixed with 2 µg HPLC-purified, anchored oligo(dT) + T7 promoter (5'-GGCCAGTGAATT-GTAATACGACTCACTATAGGGAG-GCGG-T₂₄(ACG)-3') (Eurogentec, Belgium), 40 U RNaseOUT™ (Invitrogen), and 0.9 M D(+)-trehalose (Sigma, Bornem, Belgium) in a total volume of 11 µL and heated at 75°C for 5 min. The following was added to this mixture to give a 20 µL final volume: 4 µL 5× first-strand buffer (Invitrogen), 2 µL 0.1 M DTT, 1 µL 10 mM dNTP mixture, 1 µL 1.7 M D(+)-trehalose, and 1 µL 200 U SUPERSCRIPT II (20 µL final volume). The sample was then incubated in a Uno II thermal cycler (Biometra) at 37°C for 5 min, 45°C for 10 min, and 10 cycles of 60°C for 2 min and 55°C for 2 min. To the first-strand reaction mixture, 33.4 µL 5× second-strand synthesis buffer (Invitrogen), 3.4 µL 10 mM dNTP mixture, 1 µL 10 U/µL *E. coli* DNA ligase (Invitrogen), 4 µL 10 U/µL *E. coli* DNA polymerase I (Invitrogen), and 1 µL 2 U/µL *E. coli* RNase H (Invitrogen), 103.8 µL water were added and incubated at 16°C for 2 h. The synthesized dsDNA was purified with a QIAquick kit. Antisense RNA was synthesized in a total volume of 20 µL using an AmpliScribe T7 High-Yield transcription kit (Epicentre, Madison, WI, USA), according to the manufacturer's instructions. The RNA was then purified with an RNeasy® purification kit (Qiagen). From this mixture, 3 µg amplified RNA were labeled and purified, using random nonamers in the standard protocol previously described (Genset, Paris, France).

Exponential Amplification by SMART™ cDNA Synthesis and PCR

The SMART method is based on intrinsic terminal transferase activity of a point mutant RNase H reverse tran-

scriptase. When the reverse transcription reaches the end of the mRNA template, it automatically adds a stretch of C-rich sequence to the ends of newly synthesized strands of cDNA. An oligonucleotide with a G stretch at its 3'-end could hybridize to the C stretch, creating an extended template for the reverse transcription. cDNA synthesis continues to the end of this oligomer. Second-strand cDNA synthesis is then easily accomplished using PCR with the G-extended primer and an oligo(dT) primer.

Fifty nanograms of total RNA were reverse-transcribed in 20 µL, according to the manufacturer's protocol for the SMART PCR cDNA synthesis kit (BD Biosciences Clontech), with an anchored poly(dT) primer (CDS) and a SMART II primer. Three microliters of the reverse transcription reaction were added to 100 µL PCR mixture (1× Advantage® 2 PCR buffer, 0.2 mM dNTPs, 30 nM PCR primer, 1× Advantage 2 polymerase mixture containing 40 pmol CDS and SMART PCR primers). Amplification was performed in a Uno II thermal cycler at 95°C for 1 min, 17–25 cycles of 95°C for 25 s, 65°C for 40 s, and 68°C for 6 min. To determine the optimal number of PCR cycles, 6-µL aliquots were removed from the reaction every second cycle after the first 17 cycles and analyzed on a 1.5% agarose gel. After optimization, we performed two 100-µL reactions using the same parameters and optimal number of cycles to get amplified cDNA. After purification with QIAquick, the products were labeled in two 60-µL reactions using asymmetric amplification containing 1× Advantage 2 PCR buffer, 0.2 mM d(A/T/G)TPs, 0.04 mM dCTP, 0.05 mM Cy3-dCTP or Cy5-dCTP, 60 nM dT₂₅(A/C/G) primer, and 1× Advantage 2 polymerase mixture in a Uno II thermal cycler at 95°C for 1 min, and then 10 cycles of 95°C for 25 s, 50°C for 40 s, 62°C for 3 min, and 68°C for 5 min. The amplified and labeled probes were purified using QIAquick columns.

Array Hybridization and Posthybridization Processes

The probes were resuspended in 30 µL hybridization solution (50% for-

amide, 5× SSC, 0.1% SDS, 100 µg/mL salmon sperm DNA) and prehybridized at 42°C for 30 min to block hybridizations of polyA/T tails of the cDNA on the arrays by adding poly(dA) for the direct labeling of RNA during reverse transcription and SMART or poly(dT) for the in vitro transcription hybridizations. Mouse COT-1 DNA® (1 mg/mL; Invitrogen) was added to the mixture and placed on the array under a glass coverslip. Slides were then incubated for 18 h at 42°C in a humid hybridization cabinet (Amersham Biosciences). Posthybridization washings were performed for 10 min at 56°C in 1× SSC, 0.1% SDS, twice for 10 min at 56°C in 0.1× SSC, 0.1% SDS, and for 2 min at 37°C in 0.1× SSC.

Scanning and Data Analysis

Arrays were scanned at 532 and 635 nm using a Generation III scanner. Image analysis was performed using ArrayVision (Imaging Research, ON, Canada). Spot intensities were corrected for background and filtered based on two standard deviations above the background. Ratios were normalized by a linear regression between $\log_{10}$ ratio (Cy5/Cy3) and $\log_{10}$ total intensity of Cy5*Cy3. For duplicate spots, the average ratios of Cy5/Cy3 were used for further analysis. Scatter plots were visualized using <http://www.Spotfire.net> (Spotfire AB, Göteborg, Sweden) and Statview (SAS Institute). The correlations and *P* values were calculated using Statview.

Single-Gene RT-PCR and Southern Blot Analysis

Semiquantitative RT-PCR was performed similarly as described in Huntsman et al. (9). Briefly, 100 ng mouse spleen and testis mRNA were reverse-transcribed using specific primers complementary to the 3'-end of the coding region of the desired gene with the SUPERScript II system in total volume of 30 µL. Gene-specific primers used for the amplification of the desired clones were combined with 0.5 µL reverse transcriptase mixture as template in total volume of 50 µL. To avoid overamplification, the optimal number of PCR cycles was determined by agarose gel

electrophoresis and ethidium bromide staining (18–26 cycles were performed according to the abundance of the appropriate mRNA). PCR products were separated with electrophoresis using 2% agarose gel and then transferred onto nylon membranes (Amersham Biosciences) by capillary action. ³²P-labeled probes were synthesized with PCR using plasmids with the corresponding cDNA insert that were spotted on the array with M13 forward and reverse primers in a 100 µL total volume containing 1× PCR buffer (50 mM KCl, 10 mM Tris-HCl, pH 8.5, 0.1% Triton® X-100), 1.5 mM MgCl₂, 125 µM each dNTP, 5 µCi [α -³²P]dCTP (3000 Ci/mmol), 50 pmol each primer, and 3 U Platinum *Taq* DNA polymerase (Invitrogen). Labeled PCR fragments were purified with QIAquick, resuspended in hybridization buffer, denatured, and applied onto the membrane. Hybridizations and

washing steps were performed using standard protocols (18). Membranes were exposed to phosphorimager

Figure 1. Autoradiograph showing inconsistent expression. Autoradiograph of 12 genes analyzed by semiquantitative RT-PCR between testis and spleen. Five of these genes showed inconsistent or contradictory expression ratios among the methods tested. (A) The following genes are shown: lane 1, AA049537; lane 2, AA050371; lane 3, AA058055; lane 4, AA175441; and lane 5, W13718. (B) As a control, the expression of the GAPDH gene is shown. T, testis. S, spleen.

Figure 2. Reproducibility of spleen-versus-spleen microarray analysis performed by three different labeling methods. For each method, mouse spleen RNA was labeled with Cy5 and Cy3 separately and hybridized together. The figure shows scatter plots of $\log_{10}$ (Cy5/Cy3) ratios of all 9192 spots (two replicate spots/gene) against $\log_{10}$ (intensity of Cy3*Cy5). (A) Standard labeling starting with 2 µg poly(A) RNA. (B) IVT starting with 3 µg total RNA. (C) SMART starting with 50 ng total RNA. Only ratios that had fluorescent signals significantly above background were considered for spots. No further selections were made.

screens (Amersham Biosciences), and intensity ratios were calculated with ImageQuant 4.2 software. ^{32}P -labeled products were normalized to amplified levels of the ubiquitously expressed glyceraldehyde 3-phosphate dehydrogenase (GAPDH). Figure 1 illustrates the images after autoradiography.

RESULTS AND DISCUSSION

The accuracy and sensitivity of DNA microarray analysis is a very important consideration in monitoring the transcriptome. Indeed, small changes in expression levels could be of great value in studying biochemical or other bi-

Figure 3. Reproducibility and preservation of the differential expression levels of testis versus spleen among replicate experiments for each labeling method. The figures show scatter plots of $\log_{10}$ average ratio (testis/spleen) against the same results of an independent replicate experiment within each labeling method. (A) Standard labeling (STD1 and STD2); (B) linear sample amplification (IVT1 and IVT2); and (C) exponential sample amplification by SMART-PCR (SMART1 and SMART2). The only average values considered were those for which both replicate spots had fluorescent signals significantly above background and also had a less than 2-fold difference from one another. See Table 1 for the number of genes used in this comparison.

ological functions. Another challenge of microarray gene expression analysis is often the limiting amount of RNA that can be obtained from small or limited biological samples. Thus, we optimized two sample amplification techniques: a linear amplification-based in vitro transcription method and an exponential amplification-based SMART method. The major adaptation for the in vitro transcription-linear amplification was the higher temperature used during the first-strand reverse transcription. This could be accomplished by adding trehalose to prevent the denaturation of the SUPERSCRIPT II enzyme. This modification resulted in higher yields of first-strand cDNA, possibly by eliminating the secondary structures of the template (data not shown). Using 3 µg total RNA, a typical yield of 10–20 µg amplified RNA was obtained, which corresponded to greater than 200× amplification. Another critical component of the protocol was the purity of the oligo(dT) T7 primer. Only highly purified

Table 1. Two-Tailed Paired *t* Test of Testis/Spleen Ratios within and between Labeling Methods

Comparison ^a	Mean Difference	No. of Genes ^b	<i>t</i> value	<i>P</i> value	Correlation Coefficient ^c
STD1-STD2	-0.065	1194	-1.360	0.174	0.95
IVT1-IVT2	0.620	1934	0.772	0.440	0.93
SMART1-SMART2	-0.361	1740	-3.771	0.001	0.85
STD1-IVT1	0.010	1253	0.111	0.911	0.84
STD2-IVT1	-0.001	1088	-0.005	0.996	0.85
STD1-IVT2	-0.172	1366	-1.296	0.195	0.78
STD2-IVT2	-0.167	1192	-1.414	0.157	0.77
Average STD-					
Average IVT ^d	-0.097	965	-1.097	0.273	0.83
STD1-SMART1	0.199	1243	4.436	0.0001	0.84
STD2-SMART1	0.262	1078	4.292	0.0001	0.81
STD1-SMART2	-0.245	1348	-1.761	0.078	0.70
STD2-SMART2	-0.270	1175	-1.636	0.102	0.70
Average STD-					
Average SMART ^d	-0.035	940	-0.376	0.707	0.79
Hypothesized difference = 0					
^a The numbers 1 and 2 stand for the first and replicate experiment, respectively.					
^b The number of genes that gave significant fluorescent signals above background in both datasets compared.					
^c The correlation coefficients were calculated after the log ₁₀ transformation of the data.					
^d These comparisons were performed using the average expression ratios from replicate experiments. STD, standard direct labeling of RNA during reverse transcription; IVT, in vitro transcription; and SMART, exponential amplification by SMART cDNA synthesis and PCR.					

primers resulted in the appropriate yields of cDNA, and we found it advisable to compare primers from different companies. For exponential amplification, we adapted the PCR amplification and labeling procedure from the SMART PCR cDNA synthesis kit. This technique does not use random primers and omits the ligation of sequences after the second-strand cDNA synthesis. Therefore, the reproducibility of the amplification was expected to be better than other PCR amplification approaches in which the contamination of genomic DNA or poly(A)⁺ RNA can be amplified, thus leading to nonspecific hybridization. After the PCR amplification of the cDNA, the probe was generated by asymmetric PCR, resulting in only one labeled strand. This leads to a

considerable enhancement of the signal (data not shown). The optimal number of PCR cycles had to be determined in every case. More than 10% of the spots exhibited nonreproducible intensity data (more than 2.5-fold difference between the intensities from duplicate experiments) caused by the negative effect of overcycling (data not shown). We used 50 ng total RNA as starting material, and the approximately 2000× amplification rate of SMART resulted in sufficient labeled probe for two hybridization experiments.

In this work, we primarily focused on two questions: (i) “Are amplification methods reproducible?” and (ii) “Do amplification methods introduce bias in relative transcript levels?” For the first question, we compared SpleenCy5/

Table 2. Comparison of 12 Differentially Expressed Genes

GenBank® Accession Nos.	Qualitative RT-PCR ^a	STD1	STD2	IVT1	IVT2	SMART1	SMART2
AA049537	6.55	7.14	6.25	3.57	2.00	-1.25	—
AA058055	3.23	5.26	2.94	-3.85	-7.69	—	-5.26
W82946 ^a	—	—	—	-16.67	-14.29	-7.69	-20.00
AA050371	-2.94	-2.70	-2.44	-12.50	-11.11	—	-6.67
AA118976	-2.38	-3.70	-8.33	-1.61	1.56	3.45	2.04
W36382	5.15	1.06	1.15	4.35	9.09	1.61	3.45
W47799 ^a	—	100.00	—	—	50.00	90.90	30.30
AA175329	-20.00	-11.11	—	-50.00	-50.00	-33.33	-20.00
AA175441	-12.50	—	—	-10.00	-12.50	-5.56	-7.69
W13718	1.59	-6.25	-4.35	1.32	1.41	1.43	2.22
AA124396	1.85	1.03	1.10	4.55	4.35	3.03	5.03
AA011839	-4.17	—	—	-11.11	-10.00	-11.11	-9.09
Correctly classified (bold)	—	6	5	7	6	4	5
Incorrectly classified	—	2	2	3	4	4	4
No Data	2	3	5	1	0	2	1

Comparison of differentially expressed genes from mouse testis and spleen showed inconsistent or contradictory expression ratios among labeling methods between microarray analysis and semiquantitative RT-PCR. Negative values are underexpressed ratios.

^aIn the cases of W82946 and W47799, no amplification products were obtained from either spleen or testis RNA by quantitative RT-PCR.

SpleenCy3 ratio distributions between each method (Figure 2) and compared TestisCy5/SpleenCy3 replicate experiments within each method (Figure 3). For the second question, we compared directly the TestisCy5/SpleenCy3 of the amplification methods with the standard labeling procedures (Figures 4 and 5). In addition, 12 genes with ratios that were inconsistent or contradictory among the three methods were verified via quantitative RT-PCR (see Figure 1).

The reproducibility was very high for the direct labeling of RNA during reverse transcription and in vitro transcription but slightly lower for SMART. For SpleenCy5/SpleenCy3 hybridizations, over 99% of the spots showed a Cy5/Cy3 ratio that was within a 1.5-fold variation from the expected mean; for in vitro transcription, this was 96%, and for SMART, 84%. Using standard reverse transcription and in

vitro transcription protocols, approximately 0.3% of the clones have a ratio greater than 2 or less than 0.5, while in the case of SMART, up to 1.5% of these outliers were observed. This could also be observed in the spleen/testis hybridizations. These hybridizations were repeated for each method, starting with labeling a different batch of RNA extracts from BD Biosciences Clontech. The correlation coefficients for Cy5/Cy3 ratios among the repeats were high for all three methods (Table 1). However, using a Paired *t* test, SMART replicates were significantly different statistically. Overall, SMART scored lower in these comparisons than the standard reverse transcription and in vitro transcription and was highly dependent on a careful optimization of the number of PCR cycles in each experiment.

Although the reproducibility was

high within each method, the direct comparison between labeling strategies did reveal different testis/spleen ratios and different absolute fluorescent intensities for some genes. Comparing testis/spleen ratios of the amplification methods to the standard reverse transcription again showed lower correlation coefficients than among repeats within each method (Table 1). The correlation coefficients among the methods ranged from 0.77 to 0.85 for the comparison between in vitro transcription and reverse transcription and from 0.70 to 0.84 for the comparison between SMART and reverse transcription. Correlation coefficients between in vitro transcription and reverse transcription were not statistically significant, but they were between the SMART and reverse transcription protocols depending on the repeat. Focusing on the greater than 2-fold differen-

tially expressed genes, in vitro transcription and SMART, respectively, did not detect 20%–23% of the total number of differentially expressed genes measured by reverse transcription (Figure 5). Otherwise, there were 16–33 more differentially expressed genes determined by the amplification methods. Another important observation was that absolute fluorescent intensities were different among the three methods (Figure 6). This difference in absolute fluorescence was consistent among the repeat experiments and therefore likely to be dependent on the method used. However, it did not immediately affect the expression ratios as long as the same amplification method was used for all samples tested. Interestingly, the fraction of spots with fluorescent signals significantly above background was different among the various methods, being 12%–16% higher for in vitro transcription and SMART (data not shown).

Because the standard labeling method was not necessarily the best method to conserve the initial transcript ratios, a set of genes that did not show consistent testis/spleen transcript ratios among the three methods was selected for confirmation by quantitative RT-PCR (Table 2). The results could be grouped by (i) correctly classified differential expression (ratio of microarray data was in agreement with quantitative RT-PCR), (ii) incorrectly classified differential expression, or (iii) missing data (no fluorescent signals above background). Two clones did not result in RT-PCR products, probably due to suboptimal primer binding. The results showed that only 2 out of 12 genes were incorrectly classified by reverse transcription, 3–4 in in vitro transcription repeats, and 4–5 in SMART repeats. Interestingly, after the in vitro transcription amplification, less missing data were observed than in the two other methods, which may mean that amplifying the starting material may increase the sensitivity of the assay.

Overall, our results showed that amplifying RNA starting material can produce reproducible microarray data but induces slight distortions in the initial transcript levels. However, for in vitro transcription, the percentage of potentially misinterpreted gene expression levels is negligible, and more data points

above the background level could be observed. Therefore, we suggest that direct labeling is preferred when micrograms of poly(A) RNA are available and that

in vitro transcription can be considered a good alternative if only a few micrograms of total RNA can be obtained or when large-scale extractions may nega-

Figure 4. Reproducibility and preservation of the differential expression levels of testis versus spleen among labeling methods. The figure shows scatter plots of $\log_{10}$ average ratio (testis/spleen) from (A) STD1 versus IVT1 and (B) STD1 versus SMART1. The only average values considered were those for which both replicate spots had fluorescent signals significantly above background and also had a less than 2-fold difference from one another.

Figure 5. Venn diagram of differentially expressed genes. Comparison of standard labeling (STD), linear amplification (IVT), and logarithmic amplification (SMART) was made. The total number of differentially expressed genes per method is given between brackets. A 2-fold threshold allowing a 10% window determined the differentially expressed genes.

Figure 6. Microarray results for each labeling method. Amplification of RNA starting material induces changes of the absolute fluorescence intensities after microarray analysis compared to non-amplification. The images represent the same portion of a microarray hybridized with testis RNA labeled in Cy5. As an example, some spots exhibiting significantly different absolute fluorescence intensities among the techniques are boxed.

tively affect the RNA quality. SMART amplification can be used when the RNA is really limiting, such as in the case of cells obtained after cell sorting or laser capture microscopy. However, using different labeling or amplification strategies within one experiment should be avoided to retain the high reproducibility between the samples. Nevertheless, it is clear that the sooner robust methods become available that can label small amounts of RNA without amplification, the better.

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Address correspondence to:

Dr. Paul Van Hummelen
Microarray Facility, Flanders Interuniversity
Institute for Biotechnology
Onderwijs en Navorsing, Herestraat 49
B-3000 Leuven, Belgium
e-mail: paul.vanhummelen@vib.be

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