INTRODUCTION

In DNA microarray technology for each hybridization, a mixture of two fluorescent labeled probes is usually applied, where one labeled probe is obtained from a control (untreated, unaffected) and the other is from a treated or affected sample (5). This direct comparative hybridization method allows a quantitative comparison of the relative abundance of individual sequences, although experimental variation introduced by the uneven incorporation of labels, differences in hybridization, washing, and reading often occurs (3,5). It can cause discrepancies in relative and accurate comparisons of separate experimental results, especially when they are performed by different research laboratories or at different times. Many efforts have been made to minimize these variations by purchasing accurate hybridization chambers sold by different companies, although they cannot solve the problems caused by the incorporation and uneven distribution of samples on microarrays. While kits are available to optimize the labeling step, there is no consensus, even in protocols applied by different groups. The most reliable approach to overcome these problems is the indirect comparison of signal intensities. In these kinds of experiments, each mRNA sample is compared to a reference mRNA pool composed of an equal mixture of all experimental RNA targets. The importance of this approach was first suggested by Eisen and Brown (2) and used in the comparison of different cancer cell lines (7) and different non-Hodgkin’s lymphomas (1). The application of a common reference RNA pool in each cDNA microarray experiment allows the relative gene expression in multiple samples to be analyzed. It is very difficult to obtain reliable reference RNA not only because of its need for complexity but also because of the large quantity. Today, universal reference RNA is commercially available but is extremely expensive for routine use. To obtain reference RNA from pooled, high-quality RNA, RNA isolation should be performed from cell lines representing different tissues or from different tissue samples. However, this is costly and time consuming if one wants to obtain milligrams of highly purified RNA.

We developed a novel amplification technique based on PCR amplification and a modified version of an in vitro amplification (http://www.microarrays.org/pdfs/ModifiedEberwine.pdf) to obtain high-quality reference RNA in bulk amounts, starting from micograms of mixed total RNA. Amplification of mRNAs without distortion of their initial ratios was previously presented (6). Both antisense and sense amplified RNA can be synthesized with this method and can be used as standards in diverse microarray studies.
MATERIALS AND METHODS

Construction of cDNA Microarrays

PCR-amplified cDNA fragments were spotted in duplicate on aminoalkyl-silane-treated microscope slides (Silane-Prep™ Slides; Sigma, St. Louis, MO, USA) using a MicroGrid Total Array System printer (BioRobotics, Cambridge, UK). The cDNA clones were obtained from mixed libraries, cloned in pBluescript® SK II- (New England Biolabs, Hertfordshire, UK) or pGEM® (Promega, Madison, WI, USA) plasmids with standard cloning techniques. The cDNA inserts were amplified with vector-specific primers, purified with the Montage™ PCR96 Cleanup Kit (Millipore, Bedford, MA, USA), resuspended in 50% DMSO/water, and loaded into a 384-well format microplate. Post-processing of DNA arrays was performed as described previously (6).

Reference cDNA Synthesis

Thirty-three microliters pooled total RNA (80 µg) from different organisms were reverse transcribed in a total volume of 60 µL. For the synthesis of antisense reference RNA, total RNA was mixed with 3 µL (100 pmol/µL) T7T25V and 3 µL (100 pmol/µL) FOR primers, and 3 µL (20 U/µL) RNasin® (MBI Fermentas, Vilnius, Lithuania), heated to 75°C for 5 min and cooled on ice. To this mixture, 12 µL 5× first-strand buffer (MBI Fermentas), 3 µL 10 mM dNTP mixture, and 3 µL, 200 U/µL RNase H- point mutant Moloney murine leukemia virus (MMLV) reverse transcriptase (MBI Fermentas) were added and incubated at 42°C for 2 h. For the production of sense reference RNA, the same protocol is used as for cDNA synthesis, but REVt25V and T7FOR primers were used instead of T7T25V and FOR primers. After cDNA synthesis, 240 µL TE (10 mM Tris-HCl, pH 7.5, 0.2 mM EDTA) were added.

Exponential Amplification of Reference cDNA

From the first-strand cDNA synthesis reaction mixture, 0.5 µL were amplified in a total volume of 100 µL PCR with REVt25V and T7FOR, or REVt and T7FOR primers in case of sense reference RNA production, or T7T25V and FOR, or T7T and FOR primer pairs in case of antisense amplified RNA production. The reaction mixture contained 50 mM final concentration of each primers, 1x PCR buffer (Amersham Biosciences, Piscataway, NJ, USA), 2.5 mM dNTPs, 5× PCR buffer (MBI Fermentas), 200 U/µL RNase H- point mutant Moloney murine leukemia virus (MMLV) reverse transcriptase (MBI Fermentas), and 200 U/µL RNase H- point mutant Moloney murine leukemia virus (MMLV) reverse transcriptase (MBI Fermentas), and incubated at 95°C for 5 min. After incubation, 2 µL of the first-strand reaction mixture were added to each reaction and incubated at 95°C for 5 min. After incubation, 2 µL of the first-strand reaction mixture were added to each reaction and incubated at 95°C for 5 min. After incubation, 2 µL of the first-strand reaction mixture were added to each reaction and incubated at 95°C for 5 min.

RNA Extraction and RNA Pools

For the production of rat reference RNA, commercially available total RNA was pooled. RNA (5 µg) from each of the following tissues were mixed: liver, brain, thymus, heart, lung, spleen, testicle, ovary, and kidney. Rat embryo total RNA (35 µg) was added to this mixture. All of the RNA was purchased from Ambion (Austin, TX, USA). For human reference RNA, 60 µg Universal Human Reference RNA (pooled from 10 different cell lines representing different tissues; Stratagene, La Jolla, CA, USA) was mixed with 5 µg human heart total RNA (Ambion), 5 µg human fetal brain total RNA (BD Biosciences Clontech, Palo Alto, CA, USA), 5 µg human thyroid total RNA, and 5 µg total RNA from human carotid tissue. Total RNA from thyroid and carotid tissue were purified with NucleoSpin® RNA II extraction kit (Macherey-Nagel, Düren, Germany). For alfalfa reference RNA, 14 µg total RNA from the following tissues were pooled: flower, leaf, root, seed, and germ. This RNA was mixed with 10 µg total RNA prepared from protoplasts. For RNA purification the FastRNA® kit (BIO 101, Vista, CA, USA) was used. The quality of the extracted RNA was assessed by gel electrophoresis, as well as by A260/280 ratios.
USA), 4 U Taq DNA polymerase (Amersham Biosciences), and 200 µM dNTP. Amplification was done under the following conditions: initial denaturation at 95°C for 1 min, 22 cycles of denaturation at 95°C for 35 s, annealing at 58°C for 40 s, and extension at 68°C for 4 min. PCR products were purified with a PCR purification kit (Qiagen, Hilden, Germany), according to the manufacturer’s instructions, but eluted twice with 40 µL 1/5 Elution buffer. The concentration of the eluted dsDNA was determined by UV absorbance measurement at 260 nm. The usual yield was approximately 2 µg DNA from 100 µL PCR. Forty reactions were performed from each cDNA synthesis reaction mixture from the different pooled samples. The purified PCR products were pooled, vacuum-concentrated using an Automatic SpeedVac AS160 (Savant Instrument, Farmingdale, NY, USA) and dissolved in DEPC-treated water to a final concentration of 500 ng/µL.

**Production of Reference RNA Using In Vitro Transcription**

PCR-amplified cDNA template (20 µg) was transcribed in a total volume of 200 µL using Ribomax Large Scale RNA Production System (Promega), according to the manufacturer’s instructions. The RNA was diluted with 0.3 mL DEPC-treated water, purified using 15 columns from the RNeasy® purification kit (Qiagen), and measured spectrophotically. The usual yield was 1.5–2 mg amplified RNA, which corresponds to 150–200× amplification of the PCR-amplified DNA. For long-term storage, RNA was aliquoted (aliquots contained 30 µg amplified RNA) in the presence of RNasin (final concentration of 0.1 U/µL), dried using speed vacuum, and stored at -80°C.

**Probe Preparation and Labeling**

Amplified reference RNA (2.5 µg) was labeled with Cy3 during reverse transcription using 0.4 µM random nonamer as primer, 0.1 mM d(G/T/A)TPs, 0.05 mM dCTP (Amersham Biosciences), 20 U RNasin (MBI Fermentas), 1× first-strand buffer, 200 U RNAse H point mutant MMLV reverse transcriptase, and 0.05 mM Cy3-dCTP (Amersham Biosciences) in 20 µL total volume. The RNA, primer, and RNasin were denatured at 75°C for 5 min and cooled on ice before adding the remaining reaction components. After 2 h incubation at 37°C, the heteroduplexes were denatured and the mRNA was hydrolyzed with NaOH (250 mM final concentration) for 15 min at 37°C and neutralized with 10 µL 2 M MOPS (pH 6.0). The labeled cDNA was purified with a PCR purification kit, according to the manufacturer’s instructions, but
eluted twice with 40 µL 1/2 Elution buffer. The probes were concentrated by speed vacuum and dissolved in 20 µL hybridization buffer (50% formamide, 5× SSC, 0.1% SDS, 100 µg/mL salmon sperm DNA).

Array Hybridization and Posthybridization Processes

In case of human heart expression monitoring, prehybridization was done by adding 1 µL 4 mg/mL poly(T), 2 µL 2 mg/mL human Cot DNA (Invitrogen), and 2 µL 1 mg/mL λ DNA (MBI Fermentas) to the hybridization mixture. For hybridization of labeled reference cDNA, 2 µL 1 mg/mL λ DNA and 2 µL 1 mg/mL salmon sperm DNA were added. The probe mixture was incubated at 42°C for 30 min after denaturation by heating for 5 min at 80°C. Twenty microliters of the mixture were placed on the blocked array under a 24 × 32 mm coverslip (Menzel-Glaser, Germany). To the edges of the coverslip DPX Mountant (Fluka) was poured to prevent evaporation. Slides were incubated at 42°C for 18 h in a humid hybridization chamber. After hybridization, the mountant was removed and the arrays were washed as described (6). Scanning and data analysis were performed as described previously (4). Briefly, following image analysis, genes were labeled ON or OFF according to a predetermined intensity threshold. The threshold was set at 1.5 times of the local background intensity. This cut-off level was determined from the “CH1GTB2” values (obtained from the ScanAlyze2 software, version 2.32; www.microarrays.org/software.html), which corresponds to a fraction of pixels in the spot greater than 1.5 times the background. Only if the mean spot intensity was greater than this threshold was a spot considered significantly above background. Each clone was spotted twice; thus, an average intensity could be calculated for the replicate spots.

RESULTS AND DISCUSSION

The application of common reference RNA provides an internal control and thus normalizes differences in hybridization parameters and array variations. The reference RNA should represent a mixture of different gene products with the highest possible complexity. The reference RNA, produced by our method corresponds to poly(A)+ sequences and can be prepared in bulk amounts sufficient for thousands of experiments at low cost. Mixtures of total RNA isolated from different tissues deriving from different organisms, including human, rat, and alfalfa were prepared. In case of a human total RNA pool, commercially available Universal Human Reference RNA was mixed with additional total RNA isolated from different tissues to increase the complexity of the mixture. In case of the other samples, purified or commercially available total RNA were mixed. We used these mixtures as starting material for amplification to obtain labeled sense or antisense reference cDNA.

To obtain a bulk amount of reference RNA starting from 80 µg pooled total RNA, a double amplification technique was applied. The summary of the method can be seen in Figure 1. At first, total RNA pools from different organisms were reverse-transcribed using a modified SMART™ cDNA synthesis technique (BD Biosciences Clontech). For the production of antisense probe, T7T25V and FOR primers were added.

Table 1. Sequences of Oligodeoxynucleotides Used

<table>
<thead>
<tr>
<th>Name</th>
<th>Sequence (5′→3′)</th>
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<tbody>
<tr>
<td>T7T25V</td>
<td>GGCCAGTGAATTGTAATACGACTTATAAGGGAGGCCG(T)</td>
</tr>
<tr>
<td>REVtt25V</td>
<td>TGTCTGCAGTGGTAACACCGACATCGAGTACAG(T)</td>
</tr>
<tr>
<td>T7T</td>
<td>GGCCAGTGAATTGTAATACGACTTATAAGGGAGGCCGTTT</td>
</tr>
<tr>
<td>REVt</td>
<td>TGTCTGCAGTGGTAACACCGACATCGAGTACAGTTT</td>
</tr>
<tr>
<td>T7FOR</td>
<td>GGCCAGTGAATTGTAATACGACTTATAAGGGAGGCCGGG</td>
</tr>
<tr>
<td>FOR</td>
<td>TGTCTGCAGTGGTAACACCGACATCGACCGGG</td>
</tr>
</tbody>
</table>

Figure 1. Summary of the method used in this study. A bulk amount of sense and antisense reference RNA can be obtained with combinations of exponential (PCR) and linear (in vitro transcription) amplification methods.
to the reverse transcription mixture, while in case of sense probe preparation, REVt5V and T7FOR primers were used. T7 denotes for the T7 RNA polymerase promoter sequence at the 5′-end of the primers. All the oligonucleotides used in this study were HPLC-purified (Table 1). After PCR amplification of the cDNA pool, a dsDNA mixture was generated, that had T7 promoter sequence at 5′dsDNA mixture was generated that had amplification of the cDNA pool, a HPLC-purified (Table 1). After PCR amplification of the cDNA pool, a dsDNA mixture was generated that had T7 promoter sequence at 5′- or 3′-end relative to the orientation of the original RNA. With this exponential amplification, we could achieve approximately a 400-fold improvement in the yield of the starting nucleic acid. After PCR, a second amplification, an in vitro transcription was carried out. From a DNA template with a T7 promoter at the 3′-end [relative position to the poly(A) tail of the mRNA], antisense RNA was produced that could be labeled by the incorporation of Cy5- or Cy3-labeled dUTP or dCTP using random oligonucleotides, and A. Ábrahám and R.T. Dudásné for their technical support.

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REFERENCES