Systematic Comparison of the T7-IVT and SMART-Based RNA Preamplification Techniques for DNA Microarray Experiments

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Background: Small biological samples obtained from biopsies or laser microdissection often do not yield sufficient RNA for successful microarray hybridization; therefore, RNA amplification is performed before microarray experiments. We compared 2 commonly used techniques for RNA amplification.

Methods: We compared 2 commercially available methods, Arcturus RiboAmp for in vitro transcription (IVT) and Clontech BD SMARTTM for PCR, to preamplify 50 ng of total RNA isolated from mouse livers and kidneys. Amplification factors of 3 sequences were determined by real-time PCR. Differential expression profiles were compared within and between techniques as well as with unamplified samples with 10K 50mer oligomer-spotted microarrays (MWG). The microarray results were validated on the transcript and protein levels by comparison with public expression databases.

Results: Amplification factors for specific sequences were lower after 2 rounds of IVT than after 12 cycles of SMART. Furthermore, IVT showed a clear decrease in amplification with increasing distance of the amplified sequences from the polyA tail, indicating generation of smaller products. In the microarray experiments, reproducibility of the duplicates was highest after SMART. In addition, SMART-processed samples showed higher correlation when compared with unamplified samples as well as with expression databases.

Conclusions: Whenever 1 round of T7-IVT does not yield sufficient product for microarray hybridization, which is usually the case when <200 ng of total RNA is used as starting material, we suggest the use of SMART PCR for preamplification.

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Microarray expression profiling experiments usually require RNA in larger quantities than are available from small biological samples (1, 2). Consequently, methods have been developed to amplify the RNA before its use in microarray experiments. The most frequently used method (3) introduces a 5’-terminal promoter (complementary) sequence for the T7 RNA polymerase attached to an oligo(dT) primer into the produced cDNA. After second-strand synthesis, the sense cDNA strand is used as a template for in vitro transcription (IVT).3 The RNA polymerase produces multiple copies of antisense RNA (aRNA) with amplification factors of up to 1000-fold. Because of random priming to synthesize the second strand, the IVT products are not full-length copies, and the 5’-terminal sequences of the transcripts are underrepresented in the aRNA. This procedure works well when the initial amount of total RNA is 1–3 μg and is used extensively in experiments with cDNA and short-oligonucleotide microarrays (4, 5). When less RNA is available, the aRNA yield can be increased by a second round of amplification (6, 7).

PCR-based amplification [switching mechanism at the 5’ end of the RNA transcript (SMARTTM)] was originally developed to amplify full-length cDNAs for construction of clone libraries (8, 9), and more recently has been used in microarray experiments (10–12). cDNA synthesis is performed with an oligo(dT) primer with an attached

Nonstandard abbreviations: IVT, in vitro transcription; aRNA, antisense RNA; SMART, switching mechanism at the 5’ end of the RNA transcript; dsDNA, double-stranded DNA; Ct, threshold cycle; Gapd, Mus musculus glyceraldehyde dehydrogenase gene; UTR, untranslated region; and Pbgd, Mus musculus porphobilinogen deaminase gene.
binding site for the amplification primer. The technique takes advantage of the ability of reverse transcriptase to add stretches of dC to the synthesized cDNA and thus introduce a binding site for the second amplification primer at the 5' end, yielding products that can be amplified by PCR.

Because IVT is part of the standard labeling protocol for the Affymetrix platform, the fidelity and reproducibility of this method have frequently been analyzed with Affymetrix GeneChip assays (13–15) and self-made cDNA spotted microarrays (6, 7, 16, 17). The performance of SMART has been evaluated with cDNA-spotted (12, 18) and oligonucleotide-spotted microarrays (19). Starting from low nanogram amounts of RNA, we previously used real-time PCR to determine the amplification factors of several target sequences and found a remarkably higher power of amplification for SMART PCR than for IVT (10).

Published direct comparisons of commercial reagent sets for both of the above methods are few. In these studies, the techniques were applied under different conditions, e.g., with different amounts of starting material (16) or with unknown initial amounts of RNA (20). The application of in-house-produced cDNA microarrays makes repetition of the experiment and confirmation of the results impossible. We performed a direct comparison of 2 commercially available preamplification reagent sets based on SMART (BD Clontech) and T7-IVT (Arcturus).

Materials and Methods

For a detailed description of the materials and methods used, see the Data Supplement that accompanies the online version of this article at www.clinchem.org/content/vol52/issue6 (including the supplemental file containing the description of the materials and methods, supplemental Fig. 1, and supplemental Table 1). Briefly, total RNA from livers and kidneys of BALB/c mice was isolated according to the protocol of Chomczynski and Sacchi (21) and reverse-transcribed by use of the BD Atlas SMART Fluorescent Probe Amplification Kit (Clontech Laboratories) with slight modifications. Unamplified samples for microarray hybridizations were labeled directly during reverse transcription. Amplification of 50 ng of total RNA by T7-IVT was performed with the RiboAmp RNA Amplification Kit (Arcturus). The workflow for the analysis of amplification factors is shown in Fig. 1A. PCR-based amplifications were performed with the BD Atlas SMART Fluorescent Probe Amplification Kit (Clontech). To generate Cy-labeled double-stranded DNA (dsDNA), we amplified 50 ng of total RNA for 18 cycles and labeled it with monofunctional reactive Cy dyes (Amersham).

Real-time PCR was performed, as described in the online Data Supplement (22), on the ABI7700 Sequence Detection System (Applied Biosystems) with the SYBR® Green I detection format. Relative concentrations of the target sequences before and after preamplification by IVT or SMART (i.e., the factor of preamplification) were expressed as \( e^{\Delta Ct} \), where \( \Delta Ct \) is the difference between the threshold cycle (Ct) values of the samples being compared (\( Ct_{\text{amplified}} \) – \( Ct_{\text{unamplified}} \)), and \( e \) is the determined amplification factor in the real-time PCR reactions.

Two hybridizations on 10K 50mer oligonucleotide-spotted microarrays (MWG Biotech) were performed for unamplified, T7-IVT (2 rounds)–amplified, and SMART (18 cycles)–amplified samples. The flow chart for the experimental setup is given in Fig. 1B. The data generated from the microarrays were evaluated by use of R software (http://www.cran.r-project.org/) and the limma package (23) from BioConductor (24) (http://www.bioconductor.
amplicon factors, as described in the online Data Supplement (25–27).

**Results**

**AMPLIFICATION FACTORS**

In preliminary experiments, amplification factors were determined for specific target sequences *Mus musculus* glyceraldehyde-3-phosphate dehydrogenase gene 3’ untranslated region (Gapd 3’-UTR), Gapd, and *Mus musculus* porphobilinogen deaminase (Pbgd). The results shown in Table 4 in the online Data Supplement and in Fig. 2 demonstrate a dependence of the amplification factors on the distance of the target sequence from the polyA end of the transcripts. After 2 rounds of T7-IVT, average ΔCt values between 10 and 4 were observed, which clearly decreased with increasing distance of the preamplified sequence from the 3’ end of the transcripts (ANOVA, P <0.001; Spearman r = −0.988, P <0.001). After 12 cycles of SMART amplification, for the Gapd 3’-UTR and Pbgd target sequences, the expected average ΔCt values of 12 were obtained, whereas the ΔCt value for Gapd was −8.

Additional amplification was seen for all targets after 24 SMART cycles: the average ΔCt values reached 17 for Gapd 3’-UTR and Pbgd and 14 for Gapd (Gapd showed consistently lower values than the other 2 sequences).

For a more systematic analysis of these effects, we selected 4 additional genes and determined the amplification factors of 3 target sequences, each with different distances to the polyA tail. The targets were classified according to this distance as short- (130–320 bp), medium- (760–940 bp), and long-distance (1500–1700 bp) targets, respectively. The results shown in Table 2 and Fig. 2 of the online Data Supplement confirmed the initial findings. Even after 1 round of T7-IVT, all sequences with a distance >1 kb to the polyA tail (large distance in Table 2 and Fig. 2A of the online Data Supplement) were amplified with a considerably lower efficiency than the other sequences (paired t-test on average Ct values: short vs long, P <0.005; medium vs long, P = 0.011; short vs medium, P = 0.36). This effect was even more pronounced after the 2nd round of T7-IVT (P <0.005, P <0.01, and P = 0.25, respectively). In contrast, this effect was very small for the SMART-amplified samples (see Table 2 and Fig. 2A in the online Data Supplement; ANOVA, P >0.5; paired t-test, all P values >0.2).

With respect to the distances of the target sequences to the polyA tail, after 1 round of T7-IVT, we obtained an average 200-fold amplification for sequences with a distance <1 kb (short and medium). In contrast, sequences with a distance >1 kb (large) were amplified only 15-fold (Fig. 3A). After the 2nd round, the amplification factor for short-distant sequences increased to 22 000, and that for medium-distant sequences (760–940 bp) increased to 1500. Amplification of the large-distance sequences (1400–1700 bp) was not significantly greater after the 2nd round of T7-IVT (55-fold). Independent of the distances, 12 and 24 cycles of SMART gave average amplification factors of 1500 and 166 000, respectively (Fig. 3B).

It is noteworthy that 2 rounds of T7-IVT, including the incorporation of Cy-UTP in the 2nd round, yielded ~50 μg of aRNA with ~2000 pmol of dyes (40 pmol dye/μg of aRNA), whereas 18 cycles of SMART gave 1.5–2.0 μg of dsDNA with ~40 pmol of dyes (20 pmol dye/μg of dsDNA). With samples taken after 12, 15, 18, 21, and 24 SMART cycles, we found a constant efficiency up to cycle 18. The efficiency between cycles 18 and 21 was decreased and was minimal between cycles 24 and 21 (data not shown). This indicates that the SMART reaction had not reached the plateau phase after 18 cycles.

**WITHIN- AND BETWEEN-ASSAY COMPARISONS**

We assessed the technical reproducibility by evaluating the correlation between 2 duplicate microarray experiments performed independently (see Fig. 3 of the online
Data Supplement). MA-Plots of averaged M values are shown in Fig. 4 of the online Data Supplement. The correlation of the M values between the different assays is illustrated in Fig. 4.

Among the top 200 genes considered as differentially expressed, 32 were common between the assays using unamplified and T7-IVT–amplified RNA, whereas 66 were common between the assays using unamplified and SMART-amplified RNA, and 17 (see Table 5 in the online Data Supplement) were common in all 3 assays. Among the top 100 genes, 20 genes were common between the using unamplified and T7-IVT–amplified RNA and 21 between the assays using unamplified and SMART-amplified RNA. The complete findings for all possible intersections are shown in Fig. 5.

VALIDITY OF THE MICROARRAY RESULTS
We compared the 200 (100) genes with the highest odds ratios for differential expression as found by the microarray experiments with the status of differential expression of the genes from public databases. Not all genes were found in the databases because they are either not recorded or not assigned as differentially expressed between the liver and kidney. The comparisons of the results for the genes found are given in Fig. 6.

Discussion
Complex preamplification reactions may alter the relative abundance of specific transcripts and thus yield erroneous results in the subsequent microarray analyses. The IVT reaction may introduce differences caused by incom-
complete transcription attributable to secondary structures of the mRNA and the possible susceptibility of the RNA polymerase to terminate transcription at specific sequences. IVT has been shown to yield shortened products \((13, 28)\), which is particularly problematic when the probe sequences are not designed to hybridize to the 3’ proximal part of the transcript. On the other hand, PCR-based techniques such as SMART may be sensitive to changes in reaction conditions. Any small differences in amplification efficiencies may possibly lead to considerable alterations of the transcript profile. dsDNA obtained by SMART better represents full-length copies of the transcripts than aRNA obtained from IVT \((19)\) and is more stable than aRNA, consisting of both sense and antisense strands, which allows hybridization on microarrays spotted with antisense and/or sense oligonucleotides. SMART is considerably less labor- and time-intensive than T7-IVT.

One round of IVT has been reported to yield up to \(\sim 2\) \(\mu g\) of aRNA from 100 ng of total RNA \((6)\), a result that matched well with our results (data not shown), assuming that \(\sim 1\%–5\%\) of total RNA is polyA RNA, which corresponds to a 400- to 1000-fold amplification. Starting from as few as 50 ng of total RNA, 1 round of T7-IVT does not yield sufficient labeled material for microarray experiments. Furthermore, after 1 round of IVT, preferential amplification of sequences closer to the polyA tail was already recognizable. Sequences more than 1 kb away from the polyA tail were particularly susceptible to low amplification (see Fig. 2 and Table 2 in the online Data Supplement).

Two rounds of IVT yielded \(\sim 50\) \(\mu g\) of aRNA starting from 50 ng of total RNA. Corresponding to \(2 \times 10^3\)- to \(10^4\)-fold amplification of the polyA RNAs, this amount is less than theoretically expected (\(\sim 10^6\)-fold amplification). This may be explained by the fact that Cy-labeled ribonucleotides are incorporated during the 2nd round of IVT, reducing the efficiency of the RNA polymerase. Additionally, a loss-prone purification step had to be performed. Interestingly, amplification factors for specific target sequences as determined by real-time PCR were considerably lower. This may be attributable to additional amplification of RNA species other than mRNA.

IVT amplification factors for different sequences verified by real-time PCR were clearly dependent on the distance of the detected target sequence from the polyA tail of the transcript (see Fig. 3A; also see Table 2 in the online Data Supplement). The observed decreases in the amplification factors corresponded well to the ratios of 3’ to 5’ signals reported for Affymetrix arrays \((13)\). In contrast, amplification factors obtained by SMART show no correlation to the distance of the amplified sequence from the polyA tail (Fig. 3B). These results demonstrate the ability of SMART to amplify longer products than T7-IVT; these longer products are more suited for investigation of splice variants because differentially spliced exons may be located within the whole transcript sequence and are not restricted to the 3’ end.

The high intraassay correlation of the differential expression profiles generated from the unamplified material \((r = 0.86;\) see Fig. 3A in the online Data Supplement) is in line with the results obtained by Klur et al. \((29)\), who reported that a random primed PCR amplification technique was more reproducible than 2 rounds of IVT. Other authors have demonstrated higher reproducibility with 2 rounds of IVT \((7, 15, 17, 20)\) on cDNA arrays. In agreement with several published reports \((6, 16, 17, 30)\), we found that 1 round of IVT starting with \(>200\) ng of total RNA gave expression profiles that correlated very well with those of unamplified samples (data not shown). However, as already expected from the minor correlation of technical repeats, the averaged profiles of the IVT samples amplified for 2 rounds correlated only weakly with those of the unamplified samples \((r = 0.65;\) Fig. 4A). In contrast, the SMART-amplified samples correlated noticeably better with the unamplified samples \((r = 0.85;\) Fig. 4B). It is noteworthy that both comparisons show that the M values of the preamplified samples are compressed relative to the unamplified samples. This phenomenon was also reported by others \((6, 13)\). Such compression has an impact on the list of candidate genes when these are selected by the M values. Relying only on the average M values would lead to fewer candidate genes.
genes for preamplified samples than for unamplified samples. However, when appropriate test statistics are used to select differentially expressed genes, the effect of the compression is largely compensated by lower standard errors.

Many different procedures exist for the selection of genes considered to be differentially expressed, such as controlling the individual or family-wise error rates and/or applying some threshold criteria after explorative controlling the individual or family-wise error rates genes considered to be differentially expressed, such as standard errors.

The compression is largely compensated by lower standard errors. Whenever 1 round of T7-IVT is not sufficient for microarray experiments, which is usually the case when <200 ng of total RNA is available as starting material, we suggest the use of SMART PCR for preamplification.

In conclusion, we showed that starting with as little as 50 ng of RNA, SMART was superior to 2 rounds of T7-IVT with respect to the power of amplification, length of the synthesized products, reproducibility, and validity of the microarray results. Moreover, SMART is less laborious and less time-consuming, and the products are more stable. We thank Norbert Weissmann, Ralph Schermuly, and Karin Quanz for kindly providing the tissue specimens, and the Willy-Robert Pitzer Foundation for granting the Agilent Bioanalyzer and Axon Scanner. The study was supported by the Deutsche Forschungsgemeinschaft (DFG), SFB 547, Project Z1.

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