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# Reliability of T7-Based mRNA Linear Amplification Validated by Gene Expression Analysis of Human Kidney Cells Using cDNA Microarrays

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**Key Words**
Microarrays · RNA · Amplification · Gene expression · Kidney

**Abstract**
Genome wide gene expression analysis by cDNA microarrays is often limited by minute amounts of starting RNA. We therefore tested an optimized linear RNA amplification protocol using the RiboAmp® amplification kit in the setting of cDNA microarrays. We isolated mRNA from a human kidney cell line (HK-2; ATCC) and from Universal Human Reference RNA (STR; Stratagene). After performing one and two rounds of linear RNA amplification, respectively, the amplified RNAs were co-hybridized to cDNA microarrays. Linearity and reproducibility of the individual experiments were then assessed by calculating the Pearson correlation. The intra-amplification consistency showed a correlation of 0.968 for the first round, 0.907 for the second round and 0.912 for two successive rounds of amplification. If the first round was compared to unamplified material, r was 0.925. The second round amplification yielded a correlation of 0.897 if compared to unamplified mRNA. Two rounds of amplification starting from 200 pg of mRNA compared to unamplified material resulted in a correlation of 0.868. These results indicate that linear amplification using RiboAmp® kit yields amplified RNA with a high degree of linearity and reproducibility.



## Introduction

Oligonucleotide and complementary DNA microarrays provide a powerful and versatile method to study the expression of numerous genes simultaneously. This allows the description of gene expression patterns and subsequent evaluation of complex biological pathways on an unprecedented scale. The most relevant information on pathophysiology can be obtained by studying specific cell populations. This goal can be met easily in hematological research or when cell culture experiments are performed. However, gene expression analysis in samples isolated from complex tissues such as renal biopsies is more complicated. Since new methods like laser capture microdis-

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section (LCM) [1, 2] enable us to isolate cells of specific target compartments (e.g. glomeruli or renal tubular epithelial cells), a new sector was opened for investigation. The limitation of these methods is the quantity of mRNA obtained after LCM which is usually in the range of picograms to nanograms. Since microgram amounts of mRNA are necessary for array hybridization, amplification steps have to be included into the experiment protocol. Therefore in the last decade great efforts have been made to develop linear RNA amplification methods. The polymerase chain reaction (PCR) using gene-specific primers is an excellent method of amplification. However, it lacks feasibility because it cannot be performed on tens of thousands of genes simultaneously and it skews the relative abundance levels of the messages. Therefore, in 1990, van Gelder et al. [3] described a protocol for linear RNA amplification from limited quantities of cDNA. Based on in vitro transcription using T7-RNA polymerase, they were able to amplify RNA from heterogeneous populations by a factor of $10^3$. Using this approach and by performing two rounds of linear RNA amplification, Eberwine et al. [4] were able to describe gene expression in single live neurons. This method was further optimized by the introduction of a template switch primer by Wang et al. [5]. Recently however, Baugh et al. [6] showed that following these protocols a template-independent product might be generated in addition to amplification of target mRNA. It was also shown that the specificity and reproducibility of the protocols are highly dependent on the concentration of T7-RNA polymerase, the concentration of the oligo (dT) primer and of the deoxynucleotide mixes as well as the in vitro transcription time.

Recently, a linear RNA amplification kit was developed (RiboAmp®, Arcturus, Mountain View, Calif., USA) to amplify RNA from very small amounts of material, e.g. after LCM. However, to our knowledge, this kit has not been systematically tested for reproducibility and linearity so far. Therefore, we amplified mRNA using the RiboAmp® kit and tested the reliability of this method in the setting of Cy-5/Cy-3 cDNA microarray experiments [7]. In addition, to compare microarray experiments with another well-established method, we measured the expression levels of three selected genes by quantitative real-time PCR in unamplified as well as amplified RNA samples.

## Methods

All protocols as well as the raw data and the images from the microarray experiments are available at www.microarray.at

*Cells*

Human proximal tubule cells from normal kidney (HK-2) were obtained from the American Type Culture Collection (ATCC, Rockville, Md., USA) and maintained in RPMI supplemented with 10% fetal calf serum, penicillin, streptomycin and *L*-glutamine.

*mRNA Isolation*

Poly-(A)-RNA was isolated from HK cells (and used as the test sample) and from Universal Human Reference total RNA ('STR', Stratagene, La Jolla, Calif., USA – which served as reference in the array experiments) using the FastTrack 2.0 Kit (Invitrogen, Carlsbad, Calif., USA). We used poly-(A)-RNA and not total RNA as starting material to be able to calculate the amplification factors for each round independently. We isolated 18.3 μg of mRNA from $5 \times 10^7$ cells, and 8.4 μg of mRNA from 400 μg of total RNA, respectively. The quality of mRNA was assessed by agarose gel electrophoresis and spectrophotometry. On the denaturating agarose gel, the isolated mRNA showed a homogenous smear between 400 and >10 kb. The A260/280 nm ratios were 2.01 for HK mRNA and 2.05 for STR mRNA.

*Linear RNA Amplification*

Linear mRNA amplification was performed using the RiboAmp® RNA amplification kit (Arcturus) which uses an optimized T7-RNA polymerase-catalyzed reaction to amplify minute amounts of mRNA (pico- and nanograms). The general principle of this method is thoroughly explained in van Gelder et al. [3] and Eberwine et al. [4]. In brief, the protocol uses a five-step process: (A) An oligo (dT) primer containing a T7-RNA polymerase binding site at the 5′ end is annealed to the poly-(A) fraction of the RNA and a first-strand cDNA is synthesized. (B) Using random oligonucleotides and a DNA polymerase, a second strand is synthesized yielding a double-stranded cDNA (ds cDNA). (C) ds cDNA is purified using specially designed purification columns. (D) One round of in vitro transcription (IVT) using T7-RNA polymerase yields amplified RNA (aRNA). This aRNA is antisense and contains a poly-(U) tail at the 5′ end. (E) Before purification, ds cDNA is destroyed using DNase and aRNA is isolated using the same purification columns as in (C). A second round of amplification can be performed as well by adding random oligonucleotides and performing a synthesis of first-strand cDNA. ds cDNA is then yielded in successive second-strand synthesis reaction in which the oligo (dT) primer is incorporated. Finally, IVT is performed as in the first round of amplification.

We performed three types of experiments: (1) To determine the reproducibility and linearity of one round of amplification, one round of amplification on 50 ng of poly-(A)-RNA from HK cells and STR was performed. (2) To allow estimation of the reliability of the second amplification step individually, 50 ng of the amplified RNA from the first round were taken and again amplified. (3) Additionally, two successive rounds of amplification were performed starting from 200 pg of unamplified poly-(A)-RNA to mimic the amounts likely to be obtained after RNA isolation from tissue biopsy samples using LCM. All amplifications were performed in triplicates. Quality control by spectrophotometry and agarose gel electrophoresis showed good quality of RNA.

**Table 1.** Pearson coefficient r for intra-sample variability (reproducibility)

| Experiment | r ± SD |
|---|---|
| Unamplified RNA arrays | 0.963 ± 0.003 |
| 1st round self-consistency | 0.968 ± 0.003 |
| 2nd round self-consistency | 0.907 ± 0.048 |
| 2 rounds from pg self-consistency | 0.912 ± 0.029 |

All three reactions from each category (i.e. unamplified, 1 round, 2nd round and 2 successive rounds) were compared to any other reaction of the same category in a pairwise manner. The mean Pearson coefficient r ad the standard deviation of the mean (SD) were then calculated for each category.

**Table 2.** Pearson coefficient r for inter-sample variability (linearity)

| Experiment | r ± SD |
|---|---|
| Unamplified vs. 1st round | 0.925 ± 0.006 |
| Unamplified vs. 2nd round | 0.897 ± 0.026 |
| Unamplified vs. 2 rounds from pg | 0.868 ± 0.035 |

We compared each of the 3 samples of one category with each of the 3 samples of a second category and calculated the mean Pearson coefficient r of all 9 calculations ± the standard deviation of the mean (SD).

*Labeling and Hybridization to cDNA Microarrays*

The amplified RNA samples were fluorescently labeled with Cy-5 and Cy-3 dyes using CyScribe Post Labeling Kit (Amersham Biosciences, UK). HK-RNA was reverse-transcribed and labeled with Cy-5 (red), while STR-RNA was reverse-transcribed and labeled with Cy-3 (green), according to the protocol provided with the kit. Briefly, 750 ng of either mRNA (for linearity studies) or aRNA (for reproducibility studies) were mixed with 1 µl of random nonamers and then heated at 70°C for 5 min. After addition of 4 µl 5× CyScript buffer, 2 µl 0.1 *M* DTT, 1 µl CyScribe nucleotide mix, 1 µl of aminoallyl-dUTP and 1 µl of reverse transcriptase, the samples were incubated for 90 min at 42°C. Remaining RNA was destroyed by adding 2 µl of 2.5 *M* NaOH and heating to 65°C for 10 min, followed by addition of 10 µl 2 *M* HEPES buffer. The aminoallyl-modified cDNA (AA-cDNA) was then purified by ethanol precipitation and washed with 70% ethanol. After drying at room temperature for 5–10 min, the sample was dissolved in 15 µl water. Labeling was performed by dissolving the Cy dyes in 15 µl of fresh 0.1 *M* sodium bicarbonate buffer, pH 9.0, mixing the dyes with the sample and incubation at room temperature in the dark for 1 h. Addition of 15 µl 4 *M* hydroxylamine after CyDye labeling inactivated any unreacted CyDye NHS ester molecules. The labeled probes were then combined and purified using the QIAquick PCR purification kit (Qiagen GmbH, Hilden, Germany) following the manufacturer's protocol. After elution from the QIAquick purification columns with 2 × 50 µl water, 20 µg human Cot-1 DNA (Invitrogen), 20 µg poly-(A)-RNA (Sigma, St. Louis, Mo., USA) and 20 µg yeast tRNA (Life Technologies, Carlsbad, Calif., USA) were added to each sample. A Microcon 30 filter (Millipore, Bedford, Mass., USA) concentrated the hybridization mixture, which was then adjusted to contain 3.4× SSC and 0.3% SDS in a 40 µl final volume. Following denaturation at 95°C for 1 min and a 30 min cot-1-DNA pre-annealing step at 37°C, the probes were hybridized to the microarray under a 22 × 65 mm glass coverslip (VWR, West Chester, Pa., USA) at 65°C for 16 h (overnight). Thereafter the microarrays were washed with 2× SSC, 0.1% SDS (65°C, 5 min), followed by 3 min each at room temperature in 1× SSC and 0.1× SSC. We performed hybridizations in triplicate after each RNA amplification step, i.e. first round, second round and two successive rounds from picogram amounts of mRNA, and three independent hybridizations of unamplified mRNA for linearity studies.

cDNA microarrays were fabricated by the Stanford Functional Genomics Facility as described by other groups [8, 9]. Briefly, IMAGE human cDNA (ESTs) clones were PCR amplified from DNA minipreps (Qiagen GmbH) to result in PCR products 0.5–2 kb in size. Those PCR products were then robotically arrayed onto poly-*L*-lysine-coated glass microscope slides. The cDNA microarrays used for our project contained ~45,000 different spots representing ~20,000 known genes, ~4,000 ESTs with presumed function and ~18,000 ESTs without known function. In total, the ~45,000 spots represent ~31,000 human UniGene clusters. There are also ~3,000 replicate spots present on each array.

*Scanning of Microarrays and Data Analysis*

After hybridization the microarrays were scanned using a GenePix 4000B scanner, and the images were analyzed using GenePix Pro 3.0 Software (both Axon Instruments, Union City, Calif., USA). Statistical analysis was performed using Stanford Microarray Database (http://genome-www5.stanford.edu/MicroArray/SMD/) [10]. To adjust for different labeling efficiencies between samples, the microarrays were normalized to a red to green ratio of 1.0 across the array. Physically damaged spots were excluded from analysis as well as spots showing a fluorescence signal of less than 50% over background for either red or green channel. Mean red to green fluorescence ratios were calculated after background subtraction for each single spot. Red to green ratios were calculated as logarithms to the base of 2, so that overexpression of a gene showed a value of >0 and loss of expression showed a value of <0. We applied a high stringency data selection, allowing only genes with a $\log_2$-transformed red to green value of less than −1 or more than +1. Application of these filter criteria led to the exclusion of genes which showed less than a twofold increase or decrease in expression and which showed low intra-amplification consistency (data not shown) leaving approximately 500–3,500 genes for correlation analysis.

*Statistics*

Correlation calculations were carried out using Microsoft Excel®. To determine intra-sample variability (i.e. reproducibility of hybridization and amplification) all triplicate arrays hybridized with unamplified mRNA were compared to each other (a vs. b, a vs. c and b vs. c). Identical calculations were then performed using first round, sec-

ond round and two successive rounds of amplified RNA samples. The Pearson coefficient was then expressed as the mean Pearson coefficient ± standard deviation of the mean (SD) for each category of experiments (table 1).

To test for inter-sample variability (i.e. linearity of amplification), we compared each of the three samples of one category (e.g. unamplified) with each of the three samples of a second category (e.g. one round). We then calculated the mean Pearson coefficient of all nine calculations ± SEM, which led to the mean values shown in table 2. In addition, we performed a pairwise comparison of all categories applying the t-test with correction for multiple testing [11–13].

*Quantitative Real-Time PCR*

Quantitative PCR was performed to compare gene expression levels of three selected transcripts (CA9: carbonic anhydrase IX, KCNS3: potassium voltage-gated channel and CDH3: cadherin 3) from microarray experiments with quantitative real time PCR results. The primer sequences were as follows:

CA9 for: 5′-GCT GTC ACC AGC GTC GCG TT-3′
CA9 rev: 5′-CCA GTC TCG GCT ACC TCT GCT-3′
KCNS 3 for: 5′-GCT TGA AGC ATC TGT GGA GTC-3′
KCNS 3 rev: 5′-TAG GGA TAT ATA TGC ACA GCG GA-3′
CDH3 for: 5′-GGC CAC CCA ACC CAG ATG AA-3′
CDH3 rev: 5′-CCG CTG CCC TCA TAG TCG AAC-3′

20 µl of each sample were transferred in triplets to a 96-well optical reaction plate (Applied Biosystems, Foster City, Calif., USA). For gene-specific runs, 25 µl of SYBR-Green PCR Master Mix (Applied Biosystems) were applied per sample and accomplished by adding 5 µl of primer mix using the primer pairs mentioned above. For the reference gene experiments, a reaction mix containing Tris-HCl, KCl, DMSO (Sigma-Aldrich, USA), SYBR-Green (Eubio, Vienna, Austria), dNTPs (Amersham Biosciences, Piscataway, N.Y., USA), $MgCl_2$, AmpliTaq Gold® DNA Polymerase (Applied Biosystems) and a reference primer pair (36B4; a single copy gene encoding for acidic ribosomal phosphoprotein PO located on chromosome 12) were used. 96-well optical reaction plates were brought to an ABI Prism™ Sequence Detection System (Applied Biosystems) and samples were measured for SYBR-Green activity. The cycle threshold of samples and standard was determined using SDS 1.9.1. For controlling primer dimer complexes, a melting curve was implemented. The ΔΔ ct of each transcript was then transformed into $log_2$-ratios and compared to the microarray results using the Pearson correlation.

## Results

Amplified RNA generated during the in vitro transcription process by the RiboAmp® kit is shorter than the starting mRNA template. After one round of amplification the aRNA product is about 200–1,500 bp in size, while after a second round it is 200–1,000 bp (data not shown).

The first question we addressed was: *How reproducible are independently performed amplifications?* We isolated 4.5–21.3 µg of aRNA after one round of amplification when starting from 50 ng of poly-(A)-RNA. Starting from 50 ng of first-round amplified aRNA and performing a second round of amplification yielded 26.1–61.2 µg of aRNA. Thus, the amplification factors for the first round of amplification were 90–426, and 522–1,224 for the second round of amplification, respectively. Starting from 200 pg of mRNA and performing two successive rounds of amplification yielded 2.2–27 µg of aRNA (amplification factor for two rounds: 21,000–81,000).

After co-hybridization to cDNA microarrays we tested the reproducibility of single arrays by entering the $log_2$-transformed red to green ratio of the spots into a xy scatter plot in Microsoft Excel (fig. 1). When unamplified mRNA was hybridized to a cDNA microarray, the mean intra-sample Pearson coefficient was 0.963 ± 0.003. The Pearson coefficient calculated for self-consistency of the first round amplification was r = 0.968 ± 0.003, for the second round it was r = 0.907 ± 0.048, for two successive rounds from picogram amounts it was r = 0.912 ± 0.029 (table 1).

The second question we addressed was: *How linear is this amplification method?* To assess the linearity of amplified RNA we therefore compared genes from unamplified mRNA arrays with one- and two-round amplified RNA microarrays. If unamplified RNA material is compared to first round aRNA the correlation is 0.925 ± 0.006, if compared to a second round of amplification r = 0.897 ± 0.026. Finally, if one correlates aRNA amplified during two successive rounds from picogram amounts of mRNA to unamplified mRNA, the Pearson coefficient is 0.868 ± 0.035. All correlation coefficients are shown in table 2 and selected scatter plots are shown in figures 1 and 2. In addition, we performed a pairwise comparison of all groups applying t-tests with correction for multiple testing (Bonferroni correction and maxT correction) [11–13]. No gene with a p value $<0.05$ has been obtained regardless of the correction method used (data not shown). Table 3 shows the expression values of selected genes as well as p values after maxT correction comparing categories. Reverse labeling did not change the results significantly (data not shown). Comparison of microarray results of three selected genes (CA9, KCNS3, CDH3) with the results from qRT-PCR showed a high correlation between the two methods (Pearson R 0.98–0.99, table 4). Even though we used a limited number of genes for comparison studies with qRT-PCR, the correlation with data obtained from arrays was quite satisfactory.

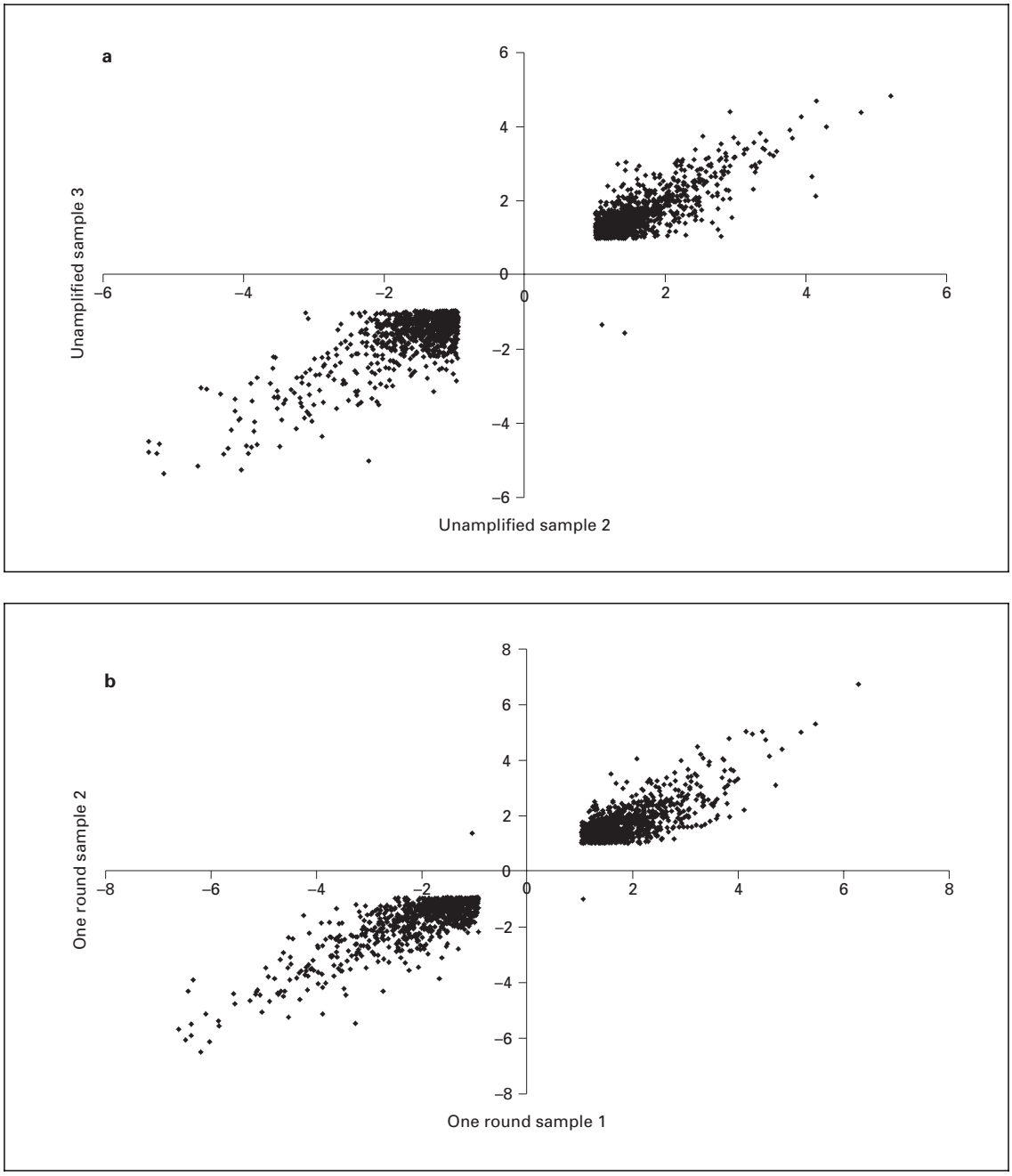
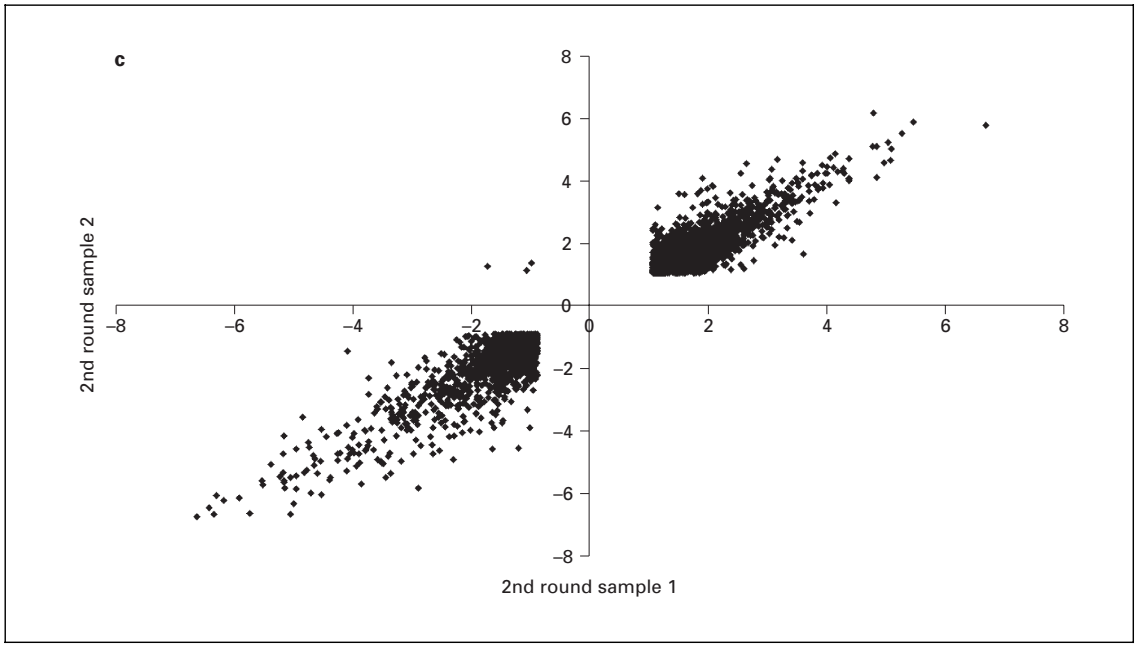
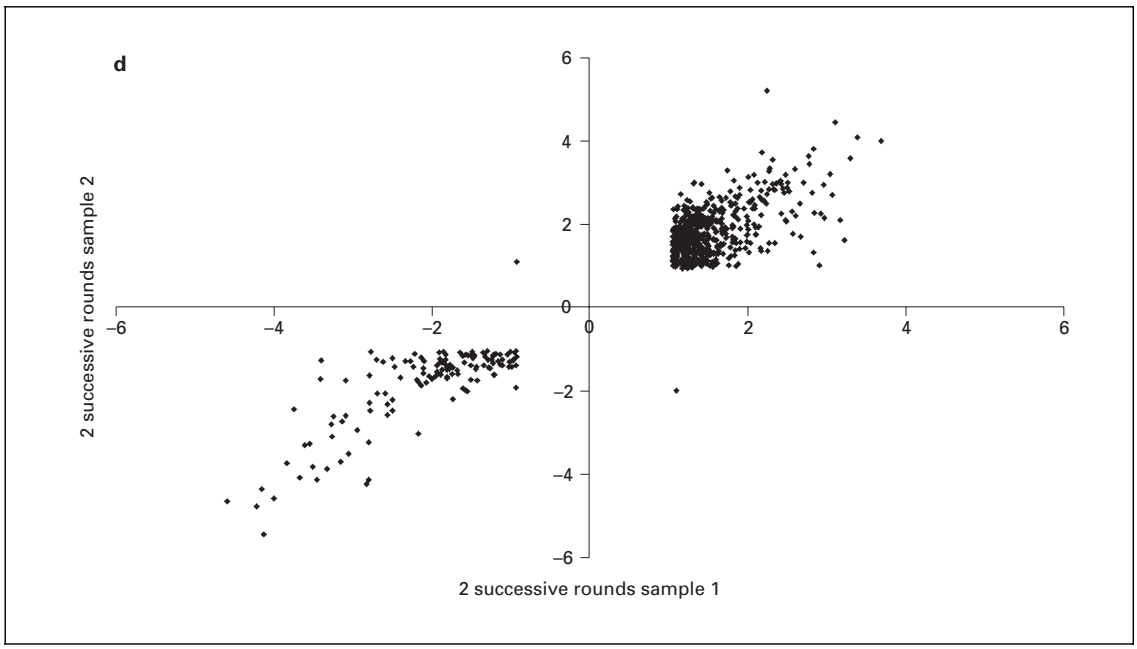



**Fig. 1.** Scatter plots of intra-sample comparisons (reproducibility testing): **a** unamplified sample 1 vs. 2, **b** one round amplified RNA 1 vs. 2, **c** 2nd round amplified RNA 1 vs. 2 and **d** two successive rounds of amplification 1 vs. 2. Not all correlations but rather a representative selection of samples is shown.

## Discussion

T7-RNA-polymerase-catalyzed in vitro transcription is to date the most reliable method for linear RNA amplification. The reliability of gene expression experiments using amplified RNA can be markedly affected by several steps of the linear amplification protocol, e.g. the incubation time of the in vitro transcription step, the concentration of dUTPs and the length of the oligo-dT-T7 primer as described by Van Gelder et al. [3]. In addition, high con-




centrations of polymerase or primers might result in the generation of a template-independent product [6]. This may not affect the expression values of highly expressed genes, but most probably compromises the results of less abundant transcripts. We therefore evaluated a commercially available, standardized and optimized RNA amplification protocol using RiboAmp® RNA amplification kit. After performing amplification without a template we did not detect any template-independent primer-specific product, which is an evidence for optimized reaction conditions (data not shown). Furthermore, when linear amplification reactions were performed using mRNA or aRNA as template, we did not detect any bands of small molecular size, which otherwise would indicate degradation or unspecific amplification. Independent amplifications of the same starting material showed a high intra-

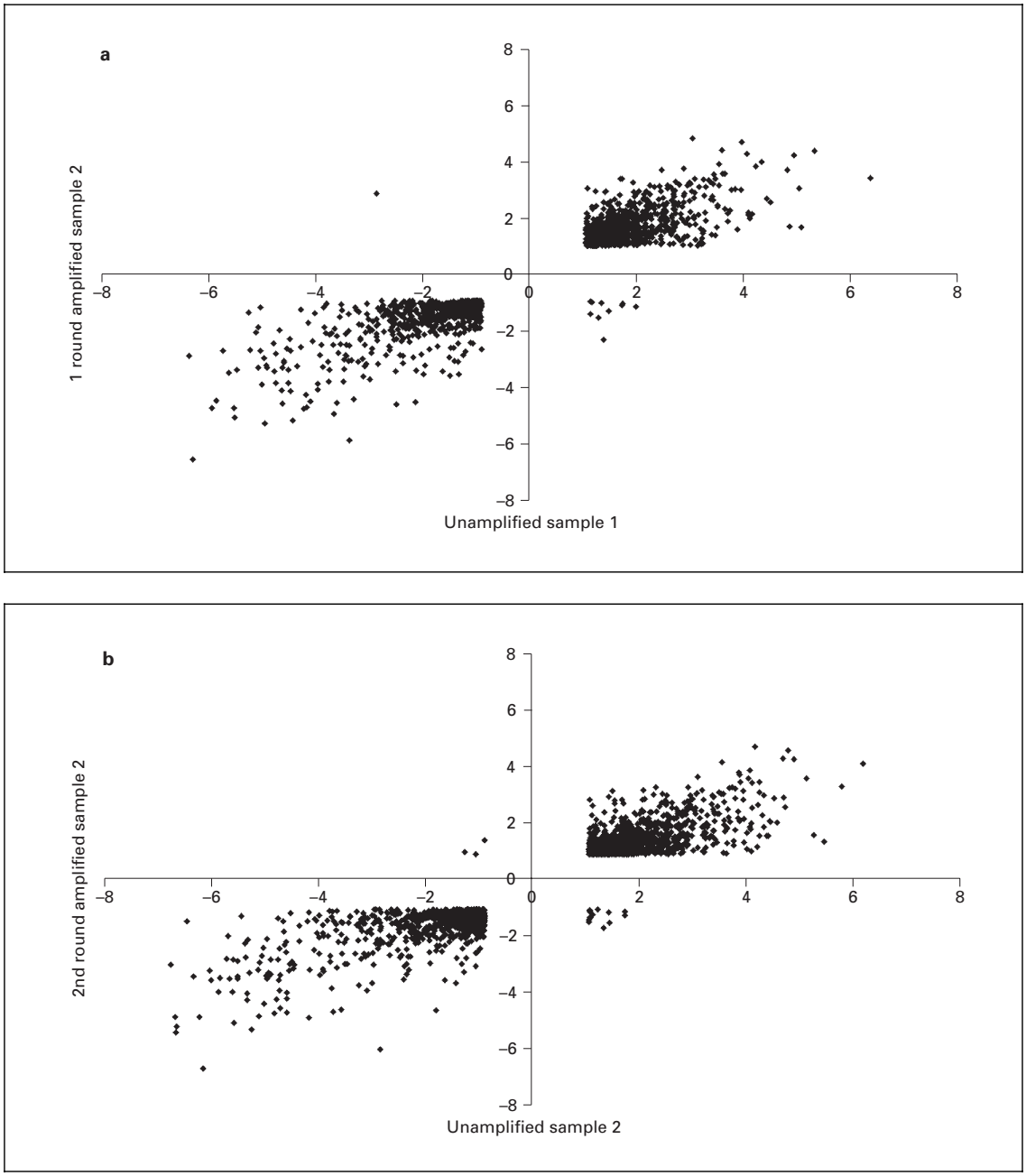



**Fig. 2.** Selected scatter plots of inter-sample comparisons (linearity testing): **a** unamplified sample 1 vs. one round amplified sample 2, **b** unamplified sample 2 vs. 2nd round amplified sample 2 and **c** unamplified sample 3 vs. two successive rounds of amplification sample 3. As in figure 1, only a representative selection of comparisons is shown.

amplification consistency (table 1). The size of the amplified RNA was shorter than the original mRNA. After amplification the amplified RNA showed a reproducible size distribution between 200 and 1,500 bp with a maximum at 300–400 bp indicating a possible underrepresentation of the 5′ end of mRNA. This is not surprising, since RiboAmp® uses random primers for second strand synthesis after reverse transcription. Therefore, this kit is not suitable for the generation of full-length ds cDNA libraries.

Because of the use of random primers during several steps of the protocol and the resulting introduction of a

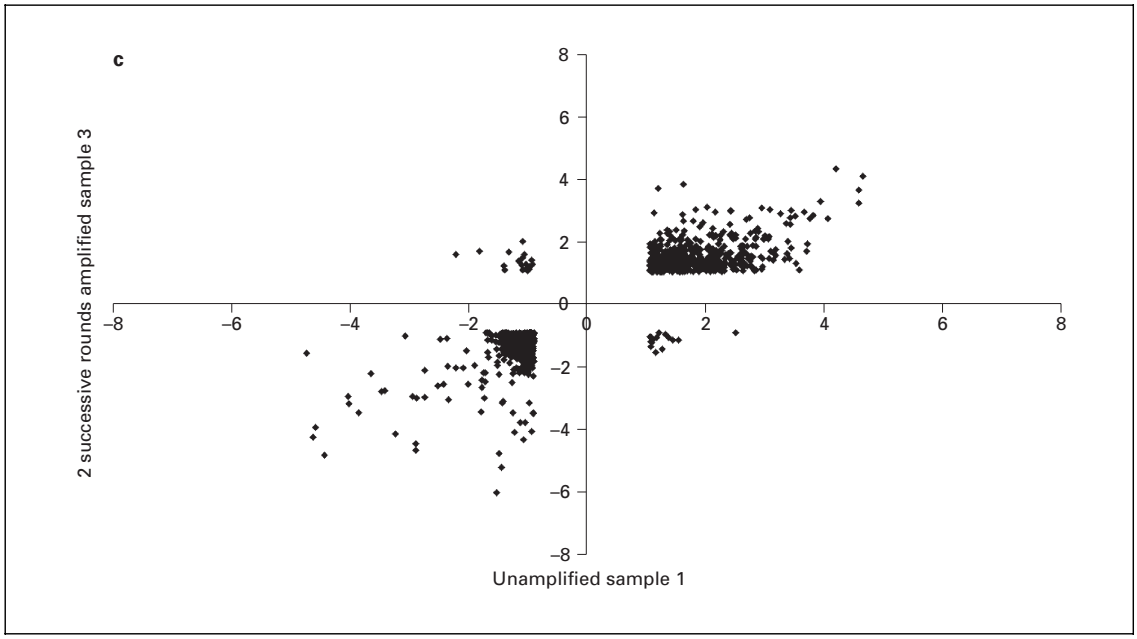



**Table 3a.** Gene expression values of selected genes before and after linear amplification[1]

| Gene | Unamplified | 1st round of amplification | 2nd round of amplification | 2 successive rounds of amplification |
|---|---|---|---|---|
| SLC4A4 | 3.34 ± 0.71 | 2.35 ± 0.44 | 2.68 ± 1.32 | 1.02 ± 0.25 |
| CA2 | 2.46 ± 0.33 | 2.26 ± 0.23 | 2.38 ± 1.09 | 1.25 ± 0.84 |
| *KCNS3* | *1.57 ± 0.18* | *2.81 ± 0.18* | *2.84 ± 1.09* | *2.80 ± 0.47* |
| SLC7A7 | 1.45 ± 0.57 | 2.56 ± 0.24 | 2.34 ± 1.08 | 1.88 ± 0.67 |
| CLDN4 | 1.40 ± 0.30 | 1.13 ± 0.09 | 0.88 ± 0.42 | 1.13 ± 1.16 |
| *CDH3* | *1.11 ± 0.22* | *1.57 ± 0.15* | *1.57 ± 0.55* | *1.60 ± 0.29* |
| MXD4 | 1.06 ± 0.18 | 1.90 ± 0.38 | 1.73 ± 0.89 | 2.30 ± 0.51 |
| COL9A3 | −0.73 ± 0.25 | −2.11 ± 0.15 | −2.82 ± 0.79 | −0.48 ± 0.52 |
| ITGA2 | −1.01 ± 0.29 | −1.06 ± 0.14 | −1.31 ± 0.06 | −0.70 ± 0.15 |
| THBS2 | −1.40 ± 0.12 | −1.57 ± 0.06 | −1.27 ± 0.40 | −0.45 ± 0.19 |
| *CA9* | *−1.50 ± 0.34* | *−1.09 ± 0.15* | *−1.39 ± 0.51* | *−0.56 ± 0.28* |
| SLC7A5 | −2.15 ± 0.10 | −2.76 ± 0.27 | −2.60 ± 0.26 | −1.68 ± 0.24 |
| IGFBP2 | −3.46 ± 0.37 | −2.80 ± 0.95 | −4.55 ± 0.35 | −3.07 ± 0.12 |
| TNFSF10 | −3.95 ± 0.24 | −4.90 ± 0.36 | −5.09 ± 0.81 | −3.00 ± 0.78 |

SLC4A4, sodium bicarbonate co-transporter member 4; CA2, carbonic anhydrase II; KCNS3, potassium voltage-gated channel delayed-rectifier subfamily S member 3; SLC7A7, cationic amino acid transporter y+ system member 7; CLDN4, claudin 4; CDH3, cadherin 3 type 1; MXD4, MAX dimerization protein 4; COL9A3, collagen type IX α3; ITGA2, integrin α2; THBS2, thrombospondin 2; CA9, carbonic anhydrase IX; SLC7A5, cationic amino acid transporter y+ system member 5; IGFBP2, insulin-like growth factor binding protein 2; TNFSF10, tumor necrosis factor (ligand) superfamily member 10.

[1] The values represent $\log_2$-transformed red (Cy5) to green (Cy3) gene expression ratios of selected transcripts in unamplified mRNA, after a first round, a second round and after 2 successive rounds of linear RNA amplification. Genes selected for validation by qRT-PCR are italicized.

3′ bias, the direct comparison of amplified and unamplified material is not recommended by the manufacturer. However, we hypothesize that using cDNA arrays which are also biased towards the 3′ end this could be ameliorated. We therefore compared amplified and unamplified material. As expected, the correlation coefficient for unamplified vs. first round amplified RNA (r = 0.925 ± 0.006) and for unamplified vs. second round amplified RNA (0.897 ± 0.026) dropped if compared to the self-consistency results. Nevertheless, the gene expression results of amplified RNA obtained from microarray hybridizations are comparable to unamplified mRNA (table 2). If one starts from low amounts of mRNA (200 pg) and performs two rounds of amplification, then the correlation of unamplified vs. amplified material is slightly worse (r = 0.868 ± 0.035). The fidelity of amplification appears to drop off with this little starting RNA as has already been shown by Baugh et al. [6]. However, after performing a pairwise comparison of all groups and applying a t-test with correction (maxT and/or Bonferroni), not a single gene appears as significantly differentially expressed between two groups – regardless of which groups are compared to each other. Interestingly, the number of genes left for analysis is somewhat lower in experiments with minute amounts of RNA to start with (data not shown). This seems to be especially true for genes with a low expression value. One possible explanation for this phenomenon may be the fact that spots with a rather low fluorescence above background on either the test or the reference sample are eliminated by our filter criteria. We therefore cannot rule out the possibility of loss of less frequent transcripts after performing double amplification when starting from low amounts of mRNA. The correlation coefficient for independently performed amplifica-

**Table 3b.** Gene expression values of selected genes before and after linear amplification[1]

| Gene | Unamplified vs. 1 round | Unamplified vs. 2nd round | Unamplified vs. 2 successive rounds |
|---|---|---|---|
| SLC4A4 | 1.0 | 1.0 | 1.0 |
| CA2 | 1.0 | 1.0 | 1.0 |
| KCNS3 | 1.0 | 1.0 | 1.0 |
| SLC7A7 | 1.0 | 1.0 | 1.0 |
| CLDN4 | 1.0 | 1.0 | 1.0 |
| CDH3 | 1.0 | 1.0 | 1.0 |
| MXD4 | 1.0 | 1.0 | 1.0 |
| COL9A3 | 1.0 | 1.0 | 1.0 |
| ITGA2 | 1.0 | 1.0 | 1.0 |
| THBS2 | 1.0 | 1.0 | 1.0 |
| CA9 | 1.0 | 1.0 | 1.0 |
| SLC7A5 | 1.0 | 1.0 | 1.0 |
| IGFBP2 | 1.0 | 1.0 | 1.0 |
| TNFSF10 | 1.0 | 1.0 | 1.0 |

See table 3a for abbreviations.

[1] p values for pairwise comparison of selected genes after maxT correction for multiple testing.

**Table 4.** Comparison of microarray results with results from qRT-PCR

| Gene | Unamplified RNA | | 1st round amp RNA | | 2nd round amp RNA | |
|---|---|---|---|---|---|---|
| | array | qRT-PCR | array | qRT-PCR | array | qRT-PCR |
| CA9 | −1.50 | −7.79 | −1.09 | −8.75 | −1.39 | −7.63 |
| CDH3 | 1.11 | 1.03 | 1.57 | −1.14 | 1.57 | −0.51 |
| KCNS3 | 1.57 | 5.17 | 2.81 | 3.20 | 2.84 | 3.59 |
| | Pearson R | 0.98 | Pearson R | 0.99 | Pearson R | 0.99 |

Three genes (CA9, CDH3 and KCNS3, see Methods section) were selected and the expression levels of those genes in HK and in STR-RNA was analyzed by quantitative real-time PCR (qRT-PCR) versus a housekeeping gene. The ΔΔ ct values were then transformed into $\log_2$ HK/STR ratios to enable correlation with microarray data. Both the microarray as well as the qRT-PCR experiments were performed in triplicates, the mean was calculated and the Pearson coefficient for linear correlation was calculated for each category (unamplified, 1st round and 2nd round). The difference of the absolute expression values between both methods is due to differences in primer and cDNA design. However, the correlation of microarray vs. qRT-PCR values shows satisfactory results (Pearson coefficients of 0.98–0.99).

tions of 0.912 ± 0.029, however, shows that even if starting from picogram quantities of mRNA, the linear amplification with RiboAmp® is highly reproducible.

Finally, we verified the microarray results of three selected genes by qRT-PCR. Although this comparison has to be dealt with great caution due to the small number of genes, the Pearson correlation between microarray and qRT-PCR results of 0.98–0.99 is quite satisfactory.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