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ORIGINAL PAPER

Reverse transcription-polymerase chain reaction on a microarray: the integrating concept of "active arrays"

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Abstract In this report we describe the proof of principle of a reverse transcription polymerase chain reaction (RT-PCR) but on-chip, with immobilized specific primers using a transcriptome of mouse-muscle fibroblasts for detection of muscle-specific expression products of these cells. The isolated total mRNA was directly incubated on an array of immobilized and solubilized specific primers, which allow the amplification of certain muscle-specific RNAs via its immobilized cDNAs. In contrast to others, the immobilized cDNA-products were directly synthesized on the chip by applying covalently bound specific primers. The products were detected by the incorporated and fluorophore-modified specific primers of the subsequently synthezised second strand. In addition, this second-strand served as a further template (like the basically used mRNA) in the subsequent solid-phase-PCR to amplify first-strand cDNA copies at the remaining immobilized specific primer-probes. This is the intrinsic factor of the amplification of certain signals of this application. The specific cDNA templates of genes coding for subunits of the mouse muscle acetylcholine receptor (Chrna1, Chrnb1, Chrnd) and the genes coding for myogenin (Myog), muscle creatine kinase (Ckmm), and ATPase (Atp2a2) were amplified on a biochip by RT-PCR directly from freshly isolated mRNA. The resulting procedure allows the detection of mRNA sequences from less than 5 pg of total RNA preparations.

Keywords Microarray · Biochip · Transcription analysis · Reverse transcription-PCR · Active array

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Introduction

Quantitative biochemistry and biology have gained new insights as a result of microarray technology. Massive parallel determination becomes possible for a multitude of binding events within one sample. Multiple measurements of a variety of species may be carried out simultaneously. In addition, it has the advantage of requiring a small amount of material and a modest investment of labor; moreover, it might save a lot of time and may easily be automated.

The power of microarrays might be enhanced, if not only binding and hybridization events are recorded, but also kinetics of binding may be followed up in each spot of the array [1]. Alternatively, the immobilized oligonucleotides of a microarray may be used as a set of primers for polymerase action. This concept, we named it "active arrays" [2, 3], has been followed by a few research teams up to now and our group has recently developed several assays on this basis. Immobilized primers are used to serve as templates for polymerases, transcriptases, etc., preserving the local information of the primary array. We demonstrated this approach for PCR [4] and for transcription [5].

Active arrays integrated in a lab-on-chip system could be a building block to solve problems of a point of care solution. The herein presented case opens the possibility of elucidating the expression profile of a small number of cells within such a system.

To overcome the difficulties in selective observation and detection of a specific expression profile embedded in the noisy cascades of signals within living organisms, it could be helpful to separate a batch of these processes from accompanying signals. To achieve defined controls and to elucidate distinct factors of synergistic interactions we follow the parallel detection, especially, of low-abundant or specific transcripts of single biological processes on an integrated biochip.

In this publication we describe the on-chip integration of biological processes to a higher level by implementation of a transcriptome analysis via on-chip cDNA-synthesis and amplification (reverse transcription polymerase chain reaction, RT-PCR [6]) using immobilized specific primers. This reverse transcription occurred at immobilized primers followed by a subsequent atypical PCR of those solid-phase-synthesized cDNAs, and using these cDNAs as immobilized single-stranded templates, whereas the products are finally immobilized also. The resulting double-stranded nucleic acids are detectable by their labeled former solubilized primers.

The parallel detection, in particular, of a specific set of low abundant mRNA species within a transcriptome is not a trivial issue. The combination of the reverse transcription and PCR is realized in the RT-PCR. RT-PCR is very sensitive and therefore the preferred method for the detection of rare mRNA molecules or small quantities of mRNA. In the following, we describe the use of immobilized primers in a microarray for detection of low abundant mRNAs by reverse transcription on the chip and subsequent amplification by PCR and combining both steps in a one-step procedure, on chip RT-PCR. Our particular on-chip RT-PCR also is a further building block in the extensive field of integration of biological processes following the prospective aim of an "artificial cell" on a biochip or for the lab-on-chip technology in modern point of care solutions.

Materials and methods

Template preparation

Total-RNA from C2C12 cells was isolated using the TRI Reagent (Molecular Research Center) according to the manufacturer's instructions. RNA quality was monitored by agarose gel electrophoresis and by use of the 260 nm/280 nm ratio. The RNA concentration was determined from the optical density at 260 nm. The total RNA was treated with DNA-free DNase I (1 u per 1 μg RNA; Stratagene, Europe) at 37 °C for 30 min, to eliminate any traces of DNA contamination. The enzymatic reaction was heat-inactivated at 70 °C for 10 min. The quantity and quality of DNase-treated RNA was determined as described above.

Primer construction

All primers were selected with regard to the NCBI GenBank sequences for the genes coding the α1, β1, δ-subunit of the mouse muscle acetylcholine receptor (Chrna1, Chrnb1, Chrnd) and the genes encoding for myogenin (Myog), muscle creatine kinase (Ckmm), and ATPase (ATP2a2) (Table 1). The forward primers were phosphorylated at the 5′-end (Roth, Germany) for immobilization. The solid-phase primers contained an additional poly(dTA)$_{10}$-spacer between the terminal phosphate group and the specific sequence. The reverse primers were labeled with a Cy3 or Cy5 modification (MWG Biotech, Germany) at the 5′-end for specific identification and direct detection of the complete amplicons on the surface.

Spotting and covalent coupling of primers

The 5′-phosphorylated primers (forward) were covalently coupled using EDC/methylimidiazole chemistry [7]. The oligonucleotides were prepared in 30 mmol L^{-1} 1-methyl-

Table 1 Primer sequences

Primer	Sequence (5′ → 3′)	Annealing region in annotated mRNA sequence
ChrnA1-for	gt aca ttg cag aga cca tga agt cag acc	1220–1248 +/+
ChrnA1-rev	aga cag acc ttc ctc tct tcc atc ttt cc	1471–1443 +/–
ChrnB1-for	tc acc tac ttc ccc ttt gac tgg cag aac	529–553 +/+
ChrnB1-rev	aac ttc ctc atg atg ccc ttc ctt ccc ac	769–741 +/–
ChrnD-for	ct aag tct cac cct ctc caa cct cat ctc	229–257 +/+
ChrnD-rev	gca cat tgc agg cat aag aaa tct gga ac	457–429 +/–
Atp2a2-for	CCT TCA GTC CTT TGA TGA GAT CAC AGC	2277–2303 +/+
Atp2a2-rev	GAA ATG ACT CAG CTG GTA GAA GGA GAC	2826–2800 +/–
Ckmm-for	cc cac aac aag ttc aag ctg aac tac aag	89–117 +/+
Ckmm-rev	ggt gac cag cct tct tga aga tct cct c	329–303 +/–
Myog-for	ag ttc ggt ccc aac cca gga gat cat ttg	576–604 +/+
Myog-rev	ctg cta cag aag tga tgg ctt ttg aca cc	788–760 +/–

imidazole (pH 6.0), 5 mg mL^{-1} EDC and 25% DMSO. The solution was directly spotted on APTES-silanized glass microscope slides or on purchased slides (Genetix, Germany) using the TopSpot microarraying robot (IMTEK, Germany). Additionally Cy5-labeled immobilization controls and negative control oligonucleotides with no sequence homologies with the target templates were spotted on each array. The spotted glass chips had to be incubated for days at room temperature. Immediately before application the biochips were immersed in 3×SSC, 0.2% SDS solution for 5 min at 65 °C, then rinsed with distilled water and centrifuged at 500 rpm for 5 min. The array was blocked by overlaying the biochip surface with blocking reagent (0.5 g succinic anhydride in 35 mL 1-methyl-2-pyrrolidinone (Aldrich, Germany) and 1.5 mL 1 mol L^{-1} boric acid, pH 8.0) for 30 min. The chips were finally rinsed with water and dried by centrifugation at 500 rpm for 5 min.

On-chip RT-PCR at immobilized primers

This RT-PCR reaction runs with a two- or one-enzyme system also.

For the two-enzyme system, initially total RNA was added to reverse transcription buffer or RNAse-free PCR buffer and 3–7 mmol L^{-1} $MgCl_2$, 500 μmol L^{-1} dNTPs, 40 u rRNasin, and 0.5 μg oligo-dT primer or 0.5 μg random hexamer primers according to the manufacturer's instructions. For concluding detection 400 nmol L^{-1} labeled reverse-primers were added to complete the solid-phase RT-PCR mixture for the second phase of the reaction. An annealing step at 70 °C for 5 min started the reaction with immediate subsequent cooling. Reverse transcriptase (ImPromII, Promega, Germany; 1 μL) and 0.5 μL of a hot-start DNA Taq polymerase, e.g. titanium Taq (Clontech, Germany) or Immolase (5 u μL^{-1}; Bioline, Germany), were added to the annealing mix. Thermal cycling was carried out as follows:

1. 25 °C for 5 min,
2. 42 °C for 1 h (reverse transcription reaction), and
3. 95 °C for 5 min for inactivation of the RT enzyme and initial denaturation for the subsequent PCR cycles with 35 cycles of 1 min at 94 °C, 1 min at 57 °C, 1 min at 68–70 °C depending on the temperature optimum of the DNA polymerase.

After the PCR reaction a final extension step at 68–70 °C for 3 min was included.

For the one-enzyme system rTth-polymerase was used, because of its ability as reverse transcriptase or thermostable DNA-dependent polymerase depending on the presence of manganese or magnesium ions. For this reaction 1× Tth buffer, 5 mmol L^{-1} $MgCl_2$, 1 mmol L^{-1} $MnCl_2$, 500 μmol L^{-1} dNTPs, 40 u rRNasin, and 0.5 μg Oligo-dT primer or 0.5 μg random hexamer primers were added according to the previously described "two-enzyme-system". Again for the concluding detection 400 nmol L^{-1} labeled reverse-primers and 1 μL rTth DNA polymerase (5 u μL^{-1}; Promega, Germany) were added to complete the solid-phase RT-PCR mixture. The reaction was started under the cycling conditions:

1. 25 °C for 5 min,
2. 60 °C for 30 min for the reverse transcription reaction, and
3. 95 °C for 5 min as initial denaturation for the subsequent PCR cycles with 35 cycles of 1 min at 94 °C, 1 min at 57 °C, 1 min at 70 °C and a final extension at 70 °C for 3 min.

The complete RT-PCR reactions were carried out in adhesive bond 25-μL volume "In situ Frames" (Eppendorf, Germany) or 35-μL volume "HybriWells" (Sigma Aldrich, Germany). The cycling was performed in a MJ PTC 200 twin tower block thermocycler (MJ Research, USA).

Detection of the PCR products on a biochip surface

After cycling the adhesive bond reaction frames were removed and the biochips were washed in 6× SSC, 0.01% SDS solution, followed by 0.05× SSC and rinsed in water for 5 min.

The PCR products which identify specific gene sequences were read out by use of a fluorescent imaging scanner (ArrayWoRx, Applied Precision, Washington, USA; or GenePix, Affymetrix, Santa Clara, USA). The Cy3-products were scanned at 595 nm and the Cy5-products at 682 nm.

Quantitative analysis of the fluorescence intensities was performed by use of ImaGene microarray analysis software (BioDiscovery, El Segundo CA, USA). Based on these data the contrast values from the spot signals were calculated according to the formula:

$$c = 100 \times (s - b)/(s + b)$$

where c is the contrast value (given in %), s is the primary signal, and b the local background in the vicinity of the spot. The signal is defined as the integral of the fluorescence intensity over the whole spot. The background value is related to an equivalent surface area.

Results

Detection of a suitable magnesium chloride concentration for the solid-phase PCR reaction

Regarding the PCR reaction itself, an increasing concentration of magnesium ions up to 7 mmol L^{-1} enhances the

efficiency of the on-chip PCR reaction dramatically. The mapped example shown in Fig. 1 represents the identified sequence of myogenin at increasing magnesium ion concentrations from 3 mmol L^{-1} to 7 mmol L^{-1}. Increasing amounts of PCR products were detected starting above 5 mmol L^{-1} magnesium ion concentration. In Fig. 1 the result for the amplification of a full gene sequence is shown. The images were visualized at 682 nm for Cy5 in the ArrayWoRx Imaging Scanner under identical scan conditions. The PCR reaction was performed with increasing concentrations of magnesium ions to 5–7 mmol L^{-1}. The PCR spots are represented in the upper threefold spot lines of each array. The lower spot lines represent the Cy5 immobilization control. The invisible control spots show the specificity of the technique.

The short-term objective of this investigation was to show that this application works well and in principle, therefore, detection limits are subjects for further studies. Nevertheless the on-chip PCR should even work beyond 7 mmol L^{-1} $MgCl_2$.

Primer density

The primers were immobilized via EDC/methylimidiazole coupling on silanixed glass microscope slides. The primer concentration in the spotting solutions was varied from 5 μmol L^{-1} to 50 μmol L^{-1}. We had found that a primer concentration of 5 μmol L^{-1} in the spotting solution was obviously sufficient for our on-chip PCR reaction with immobilized primers on glassy surfaces if supplemented with 25% DMSO (unpublished data). In the current analysis the same result (Fig. 2) was found for the on-chip RT-PCR without eliciting the detection limit, which should not be object of this study.

On the basis of contrast value, the spot evaluation showed minor differences between different primer spotting concentrations in the range from 5 μmol L^{-1} to 50 μmol L^{-1}. A concentration of 5 μmol L^{-1} primer in the spotting solution is sufficient to achieve at least a density of immobilized primers sufficient for effective polymerase elongation. The lowest effective concentration was therefore used in all further experiments.

Detection and specificity of mRNA sequences by on-chip RT-PCR

Using two enzymes (section "On-chip RT-PCR at immobilized primers") or only one enzyme for the on-chip RT-PCR reaction showed no significant differences in the results, but the "one-enzyme-system" was preferred because of the simpler handling and the lower costs.

The probes for the mRNA sequences of the genes for mouse muscle acetylcholine receptor subunits α1, β1, and δ (Chrna1, Chrnb1, Chrnd) and for the following other genes coding for myogenin (Myog), muscle creatine kinase (Ckmm), and Atpase (Atp2a2) could be detected by the described application.

In all cases the reverse labeled primer for one mRNA species was added to the reaction. The prior synthezised and immobilized cDNAs of the others served as controls,

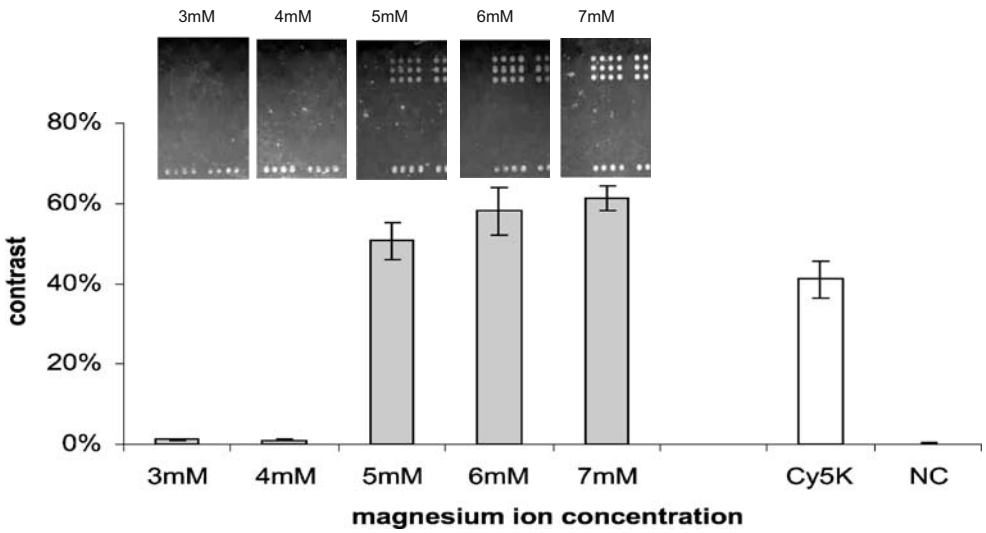


Fig. 1 Optimization of the magnesium ion concentration. The PCR reaction was performed with increasing concentrations of magnesium ions to 5–7 mmol L^{-1}. The PCR spots are represented in the upper threefold spot lines of each array. The lower spot line presents the Cy5 immobilization control. The images were visualized at 682 nm for Cy5 in the ArrayWoRx imaging scanner under identical scan conditions. The diagram below the arrays shows the mean results from analysis of the contrast values based on 24 spots for each reaction array. The columns correspond to the array above. The primers which identify other gene sequences are spotted as negative controls (*NC*) between the upper spot lines and the lower immobilization control line (blanks). The distinct specificity of the reactions is obvious; the control spots remained invisible after synthesis

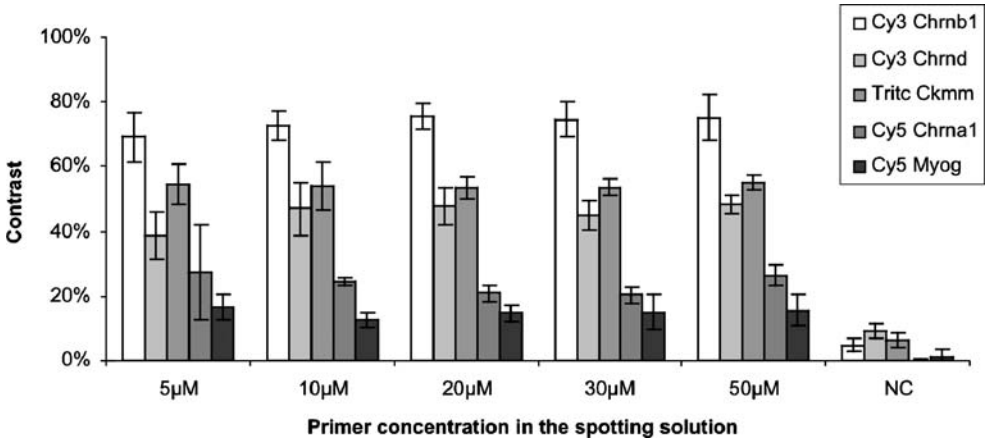


Fig. 2 Comparison of contrast data for the on-chip PCR with different primer spotting concentrations. The unscaled Tif-data of the images in Fig. 3 were analyzed by calculating the contrast of the signal over the background. Each column represents the mean of the contrast analysis based on four spots for each primer concentration

and vice versa. Two examples, Cy5 and a Cy3-primer-generated amplification of specific products are shown in Fig. 3. The reactions of the other genes showed similar results.

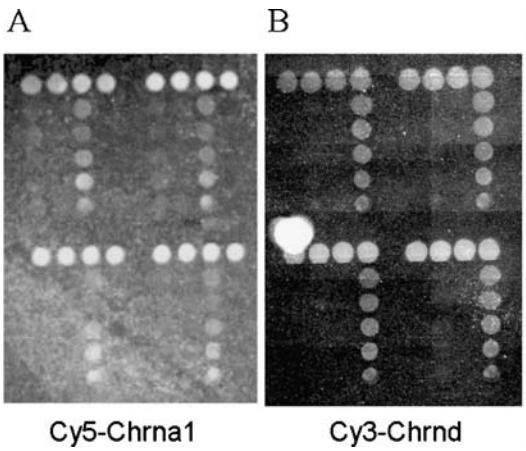
Sensitivity of the system

The amounts of template RNA used in the amplification reactions range from 5 pg up to 1 µg total RNA preparations [8]. Positive amplification signals could be detected in all the mRNA species tested here with similar results by scanning the array immediately after the RT-PCR reaction.

A showcase result for ATPase is demonstrated in Fig. 4. The arrays were detected immediately after the reaction in a fluorescence imaging scanner under constant conditions, e. g. the same gain adjustment (Fig. 4A). Additionally, the scanning data were analyzed by calculating the spot intensities and the contrast values (Fig. 4C) to confirm the images. Dilutions of total-RNA preparations used per on-chip reaction were 1 µg to 5 pg.

We should point out that the use of 5 pg RNA is not the lower detection limit. Regarding the contrast value for 5 pg RNA of about 64%, there is a sufficient range to use smaller amounts of RNA if the cut-off was set to 40%.

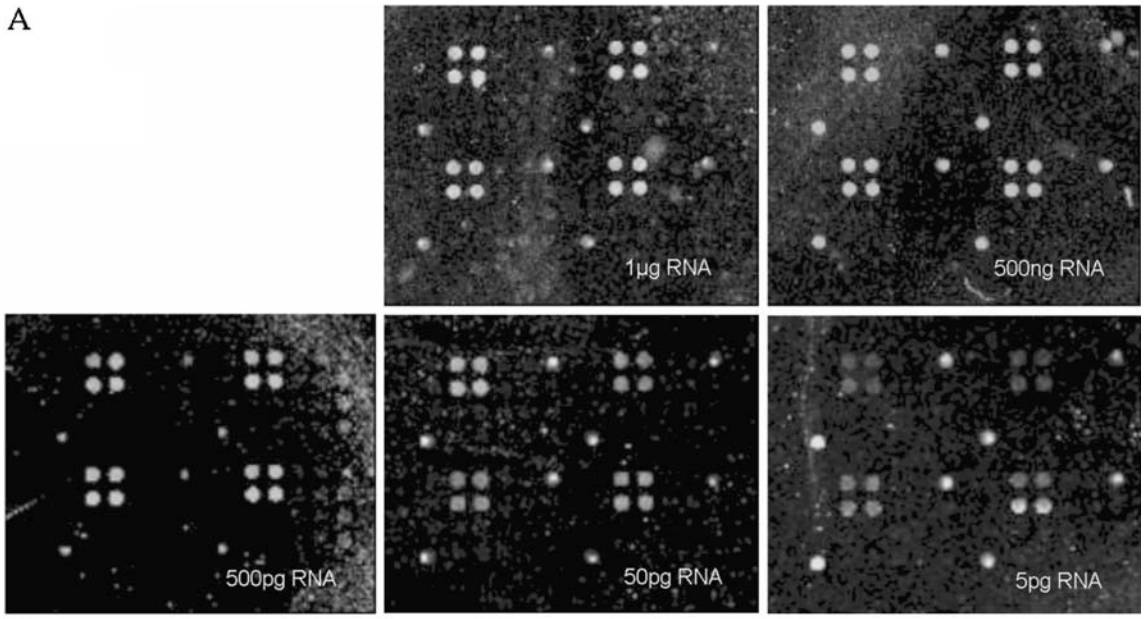
To verify the quality of the biochips each reaction array contained Cy5 immobilization control spots (Cy5K) and 10 µmol L^{-1} oligonucleotide spotting solution. The primers were spotted in a fourfold array format. Additionally, the scanning data were analyzed by calculating the spot intensities and the contrast values (Fig. 4, lower diagram) to confirm the images. All images were scanned at 682 nm for Cy5 under identical conditions using the Affymetrix Imaging Scanner at 60 db gain adjustment. The unscaled scan data were analyzed by the ImaGene software and by calculating the contrast of the signal over the background.



C

Cy5K	Cy5K	Cy5K	Cy5K
B1 50µM	M 50µM	**A1** 50µM	**D** 50µM
B1 30µM	M 30µM	**A1** 30µM	**D** 30µM
B1 20µM	M 20µM	**A1** 20µM	**D** 20µM
B1 10µM	M 10µM	**A1** 10µM	**D** 10µM
B1 5µM	M 5µM	**A1** 5µM	**D** 5µM

Fig. 3 Cy5 and Cy3 amplification products synthesized by RT-PCR at chip-immobilized primers. The *upper horizontal spot line* represents the Cy5 immobilization control, whereas the *vertical rows* identify the specific amplification products, e.g. Chrna1 (A) and Chrnd (B). The images were visualized at 682 nm for Cy5 and at 595 nm for Cy3 in the ArrayWoRx imaging scanner. Both pictures were taken in overlay mode showing the fluorescence detection of both channels (*Cy3* and *Cy5*). Abbreviations of the array layout in C: *Cy5K*, Cy5-immobilization control; *A1*, Chrna1; *B1*, Chrnb1; *D*, Chrnd; *M*, myogenin



B

A1	ATP	ATP	D	D	Cy5 K	A1	ATP	ATP	D	D	Cy5 K
A1	ATP	ATP	D	D	NC	A1	ATP	ATP	D	D	NC
A1	B1	B1	M	M	NC	A1	B1	B1	M	M	NC
Cy5 K	B1	B1	M	M	NC	Cy5 K	B1	B1	M	M	NC
A1	ATP	ATP	D	D	Cy5 K	A1	ATP	ATP	D	D	Cy5 K
A1	ATP	ATP	D	D	NC	A1	ATP	ATP	D	D	NC
A1	B1	B1	M	M	NC	A1	B1	B1	M	M	NC
Cy5 K	B1	B1	M	M	NC	Cy5 K	B1	B1	M	M	NC

C

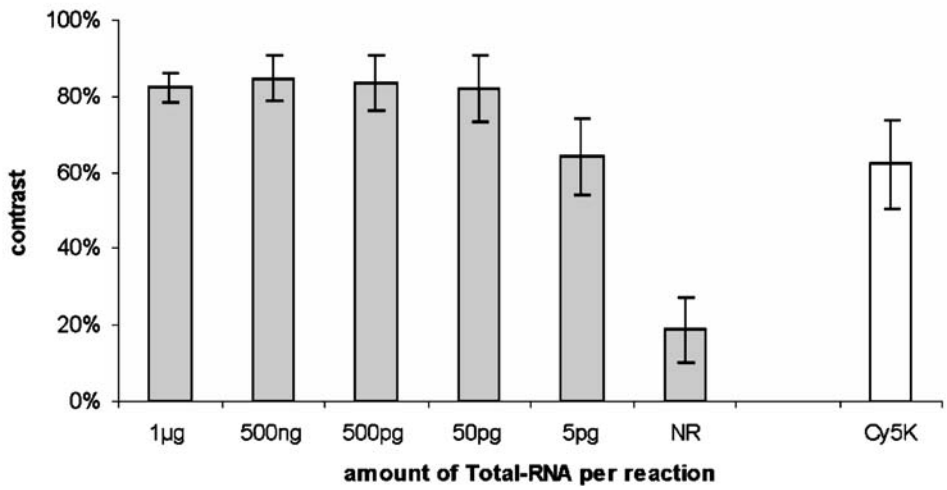


Fig. 4 Detection of diluted total RNA preparations by RT-PCR performed in a on-chip reaction. Dilutions of total-RNA preparations from 1 µg to 5 pg per on-chip reaction were used. The primers were spotted in a fourfold array format (*NR* = *NC* means the negative control reaction without RNA supplement. To verify the quality of the biochips each reaction array contained Cy5 immobilization control spots (*Cy5K*) and a concentration of the oligonucleotide spotting solution of 10 µmol L^{-1}—*A1*, Chrna1; *B1*, Chrnb1; *D*, Chrnd; M, myogenin). A. The image shows the amplification of the Atp2a2 sequence (see array layout B). All images were scanned at 682 nm for Cy5 under identical conditions using the Affymetrix Imaging Scanner at 60 db gain adjustment. B. Array layout. C. The unscaled scan data were analyzed by the ImaGene software and by calculating the contrast of the signal over the background

Discussion

This study should be understood as a proof of principle which shows that an integrated on-chip RT-PCR with immobilized primers works. This is one step within the concept of "active arrays" which could serve as suitable solutions in point-of-care applications. It should not be understood as a system competing with real-time PCR where highly parallel setups and quantitative results take center stage.

Many methods exist for analysis of gene expression. Some are appropriate for only a few sets of genes, e.g. Northern blotting. Other applications are suitable for more than thousands of genes, e.g. microarrays. All methods are based on hybridization techniques. But Northern blotting includes time-consuming steps lasting up to several days to study only a single gene in a few samples. In contrast, microarrays offer a well suited alternative for the genomic profiling of thousands of genes in one application. However, this method is laborious and cost intensive. For most applications large quantities of starting material (e.g. RNA) are required and, therefore, an amplification step has to be integrated.

The principle of RT-PCR on a solid surface was described by Hamaguchi et al. [9], who used polypropylene microtiter plates with immobilized poly-dT oligonucleotides for cDNA synthesis.

In this study the specifically synthesized and immobilized cDNAs served as templates for the subsequent specific PCR reactions.

In common studies the RT-PCR products are primarily not immobilized, e.g. Juang et al. [10], but are in solution and therefore detected by gel electrophoresis.

An approach of RT-PCR and a subsequent immobilization of the products directly after the reaction was described by Wilson and Carson [11]. They used microwells (Nucleo-Link Strips), in which the primers were immobilized. Detection of the RT-PCR products was realized by hybridization with biotin-labeled probes and a streptavidine–alkaline phosphatase assay.

In contrast with comparable investigations, in this study no subsequent hybridization was necessary and we worked with total RNA preparations from 1 μg down to 5 pg, and the low concentration was not the detection limit. More experiments with reduced RNA concentrations must be conducted to reveal the detection limit.

For on-chip-PCR application the immobilized primer is often also added as a dissolved primer for increased reaction efficiency [12, 13]. This is not necessary for the method described here, because random hexamer primers and/or oligo-dT primers were used during the cDNA synthesis to enhance the amount of template. Residual hexamers and generated short cDNAs serve as additional free primers for the PCR reaction.

Based on these results our method is practicable for analysis of genes from small amounts of RNA and therefore may be an interesting new tool for studying laser capture microdissected probes of "lab-on-chip" applications or detecting low-abundance genes.

Conclusion

Our application uses on-chip RT-PCR as a technology for detection of mRNAs from total RNA preparations. No pre-amplification step is required for this method, because the amplification and detection is integrated as an on-chip RT-PCR with immobilized specific primers. The approach detects the specific gene of interest based on a microarray spotted on a glassy surface. We minimized the detection procedure of the amplification products by incorporation of fluorescence-labeled primers during PCR cycling. The generated amplicons can be detected directly by commercial standard scanners.

The described technique is suitable for study of small sets of genes, but for a large number of samples. Therefore this method could be applicable for diagnostic analysis, which often needs to detect only a few specific genes of interest. Using this convenient strategy it must be possible to amplify immobilized templates that serve as functional RNA templates for subsequent translation of phenotypic individual proteins.

Our described method of reverse transcription of RNA in cDNA, followed by PCR of specific gene sequences and their detection on a microarray-based support, combined in a single reaction, is a promising up-to-date solution for the lab-on-chip technology and represents an active array building block for point-of-care applications.

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