



# Microfluidic-based enzymatic on-chip labeling of miRNAs

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## Abstract

Small noncoding RNAs (sncRNAs) have moved from oddity to recognized important players in gene regulation. Next generation sequencing approaches discover more and more such molecules from a variety of different groups, but flexible tools translating this sequence information into affordable high-throughput assays are missing. Here we describe a microfluidic primer extension assay (MPEA) for the detection of sncRNAs on highly flexible microfluidic microarrays which combines several beneficial parameters: it can effortlessly incorporate any new sequence information; it is sensitive enough to work with as little as 20 ng of total RNA and has a high level of specificity owing to a combination of a conventional hybridization assay and an enzymatic elongation step. Importantly, no labeling step is needed before hybridization and – because of its high sensitivity – no amplification is required. Both aspects ensure that no bias is introduced by such processes. Although the assay is exemplified with miRNAs, the flexibility of the technology platform allows the analysis of any type of sncRNA, such as piRNAs.

## Introduction

Small noncoding RNAs (sncRNAs) have emerged as an important class of regulatory RNA molecules with microRNAs (miRNAs) currently being the best characterized group [1]. They are approximately 21–23 nucleotides in length and have been established as post-translational regulators of gene expression [2]. This regulation is achieved by binding miRNA to regions of at least partial complementary in the 3'-UTRs of target mRNAs, which then leads to either interference with protein translation or a decrease in target mRNA stability [3]. miRNAs have been shown to be implicated in a variety of different biological functions, for example, they have been found to act as oncogenes and tumor suppressors [4], are involved in cardiovascular biology [5] and suppress host defense mechanisms during virus infections [6]. The less well understood Piwi-interacting RNAs (piRNAs) [7], *trans*-acting small interfering RNAs (tasiRNAs) [8], the small

RNAs (sRNAs) from bacteria [9] and other recently identified groups indicate that the field of sncRNAs is likely to expand in the near future.

Moreover, the number of known miRNA increases steadily: in early 2004, only a total of 719 entries were deposited in the central miRNA database miRBase hosted by the Sanger Institute [10–12]. This has increased to well over 6000 entries in 2008. A new release is published roughly every three to four months and not only contains additions from next generation sequencing approaches [13], but occasionally also changes in the sequences of already published miRNAs. These constant changes make it necessary to have flexible tools available to deal with the most recent and comprehensive sequence information available.

Microarrays are a versatile platform for the high-throughput analysis of known sncRNAs, currently focussing on the analysis of miRNAs. Different commercial and selfmade versions are available [14–16] and the standard analysis procedure involves the hybridization of already labeled RNA molecules. As a modification, the

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so-called RNA-primed, Array-based, Klenow Extension (RAKE) assay was developed [17]. Unlabeled RNA is hybridized to the array and labeling is performed by using the bound miRNAs as a primer for an enzymatic elongation with labeled nucleotides. The advantage of the RAKE assay is its two-tier specificity, combining the specificity of a standard hybridization assay with the high discriminatory power of enzymatic elongation. The strength of the classical hybridization assay lies in detecting mismatches in the central region of the capture probe–miRNA duplex, while the enzymatic elongation adds specificity toward the 3'-end. Good specificity toward the ends is particularly desirable, because miRNAs frequently occur in families with as little as one nucleotide difference, as in the let-7 family. An increase in discriminatory power at the otherwise difficult to analyze ends leads to more relevant biological information, because it has been suggested that different members of miRNA families are involved in different physiological processes [18,19]. In addition, the on-chip-labeling procedure also permits hybridization of unlabeled and therefore more natural RNA molecules; no dye or biotin molecule influences the hybridization behavior.

Despite its obvious benefits, the RAKE assay has so far been used only for the experimental verification of computationally predicted miRNAs [20], the profiling of miRNAs from FFPE-material [21] and an analysis of miRNA expression levels after HIV infection [22]. Recently, a bead-based enzymatic-labeling assay was presented [23]. It requires less starting material than the conventional RAKE assays but involves the PCR amplification of the target molecules, which increases the likelihood of introducing a bias. The fact that this assay has not yet found wide application might be because of the need for specially designed capture probes and the relatively large amount of starting material needed. The previously described array-based RAKE assays were all performed on standard microarray slides, requiring 4 µg of total RNA [17] or even 20 µg of sRNA [20] because of the large volumes needed for hybridization. Such amounts are difficult to obtain from samples such as laser capture microdissections (LCMs). To apply this powerful enzymatic on-chip-labeling technique to even small amounts of RNA, optimizations and modifications are necessary. Here we demonstrate the adaptation to a microfluidic microarray technology. By combination with light-directed *in situ* synthesis, only an electronic table with the sequence information is required to synthesize the capture of oligonucleotides inside the channels of a microfluidic array, thus providing the flexibility to immediately translate new sequence information into array content. Microfluidics not only have the advantage of a directed flow of liquids providing high uniformity and reproducibility, but also decrease sample volumes significantly: each reaction chamber has a capacity of approximately 3 µl [24]. This small volume reduces the sample quantity needed for appropriate concentrations and can thus be used with significantly smaller amounts of starting material. This miRNA-based microfluidic primer extension assay (MPEA) provides a high-throughput microarray platform for the analysis of any type of sncRNA with the two-tier specificity of the conventional RAKE assay, reduced amounts of input RNA and increased flexibility. We have used here the analysis of miRNAs to document its versatility and performance, but the assay can easily be adapted to any other class of sncRNA.

## Materials and methods

### Design and synthesis of microfluidic microarrays

All microarrays were synthesized and analyzed with the febit (febit biomed, Heidelberg, Germany) Geniom One technology. Eight microarrays each consisting of a meandering microchannel form the so-called 'Biochip'. Each Biochip can therefore be used to analyze eight different samples independently. The flexible oligomer synthesis is done *in situ* inside the microchannels using a light-directed process [24].

The probes were designed as the reverse complements of the mature miRNA sequences as published in the Sanger miRBase release version 11.0 (<http://microrna.sanger.ac.uk/>) plus the additional number of extra nucleotides at the 5'-end of the capture oligonucleotide as needed for the enzymatic extension. For conventional miRNA hybridization assays the reverse complement of the miRNA sequences as published in the miRBase release 11.0 were synthesized with seven intra-array replicates.

### RNA enrichment and labeling

RNA from human brain, heart, liver and skeletal muscle was obtained from Applied Biosystems (Foster City, CA, USA; First-Choice<sup>®</sup> total RNA). RNA oligonucleotides were obtained from Sigma–Proligo (The Woodlands, TX, USA). For the standard hybridization assay, total RNA in the indicated amounts was labeled using the FlashTag Biotin Kit from Genisphere Inc. (Hatfield, PA, USA) according to the manufacturer's instructions. Enrichment for molecules smaller than 200 nucleotides was done using RNeasy columns (Qiagen, Hilden, Germany) according to a modified protocol from the manufacturer. In brief, total RNA was dried in a speed-vac, resuspended in 350 µl RLT buffer plus 1 volume 80% ethanol and loaded onto an RNeasy column. The flow-through was mixed with 1.2 volumes of 100% ethanol and loaded onto a fresh RNeasy column, washed twice with 500 µl buffer RPE and eluted with 30 µl nuclease-free water.

### Microarray hybridization and detection

Hybridizations were performed using the febit external hybridization unit overnight (16 hours) at 42°C. Washing was done in the Geniom machine, including the detection of biotin with streptavidin–phycoerythrin (SAPE) and a signal amplification step using biotinylated antistreptavidin antibodies (Vector Laboratories, Burlingame, CA, USA) and a second incubation with SAPE (Invitrogen, Carlsbad, CA, USA). For the MPEA assay the stringent wash program 'febit washing (T = variable, Port = Buffer-1)' was used and all enzymatic manipulations were done using the Vybrid (febit biomed, Heidelberg, Germany) as shown in Fig. 1. After a preincubation with 1× Klenow buffer (Buffer 2, NEB, Ipswich, ME, USA), the enzymatic elongation was done with 0.25 U/µl Klenow Fragment (3'-5' exo-) (NEB, Ipswich, ME, USA) in a 1× buffer with the indicated concentration and type of biotinylated nucleotide (biotin-11-dATP, biotin-11-dCTP, biotin-11-dGTP or biotin-11-dUTP; PerkinElmer, Waltham, MA, USA) at 37°C for 15 min. The elongation was stopped using 6× SSPE buffer (Sigma–Aldrich, Munich, Germany). The biochip was then transferred into the Geniom instrument for the 'miRNA standard' washing procedure followed by signal amplification as described above. The detection was done using the appropriate filter set (Cy3) employing the autoexposure function of the

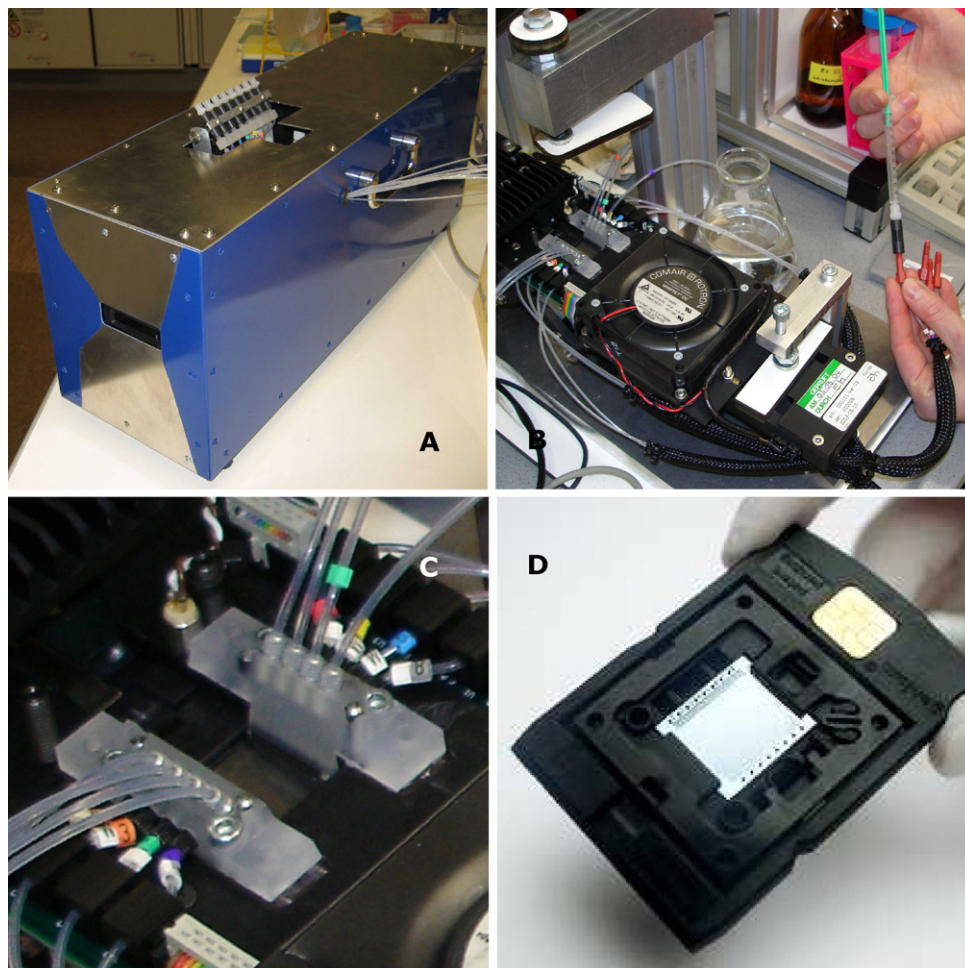


FIGURE 1

(A) Geniom Vybrid instrument for processing enzymatic reactions on microarrays. (B) Inside view showing the peltier-supported temperature control. (C) Biochip mounting with eight individual inlet and outlet tubings. (D) Microfluidic biochip, a contained system with eight independent microarrays within microchannels.

Geniom software. Feature extraction was done using the in-built Geniom feature extraction algorithm.

#### Microarray data analysis

Raw data was read into R [25] as a tab delimited text file. The data was then converted into a matrix, with rows corresponding to the features and columns corresponding to the different samples. The array background was calculated as the median signal intensity of all 'blank-control' features on the array and subtracted from the intensity values of all other intensities. Subsequently, the non-positive values were substituted with '10'. After applying quantile normalization [26] to the background adjusted data set, intra-array replicates were combined by their mean intensity.

#### TaqMan assays

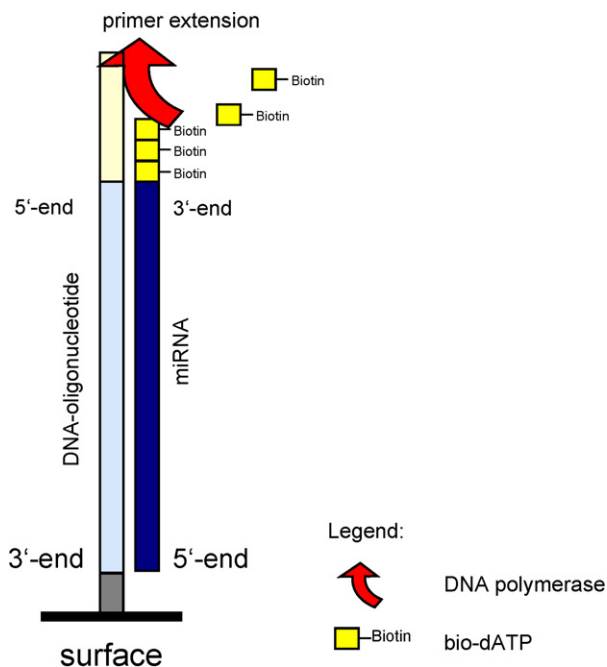
Real-time quantitative PCR was performed using TaqMan miRNA assays from Applied Biosystems (Foster City, CA, USA), according to the manufacturer's instruction. Per assay, 10 ng of total RNA was used and each analysis was performed in quadruplicates. Z30 was used as a normalization control. Data analysis was performed with the public Relative Expression Software Tool (REST) [27].

## Results

### Optimization of elongation conditions

As can be concluded from the MPEA principle shown in Fig. 2, several parameters can be investigated and optimized. These include the probe orientation (surface attachment of the capture probe at either the 3'- or the 5'-end), amount of sample used for hybridization, type, number and concentration of the biotinylated nucleotide available for the elongation step and the extension time. To have a benchmark for the optimization of the MPEA, a standard reference data set was needed. All optimization procedures were therefore compared to a reference data set obtained from a conventional hybridization assay for the analysis of miRNAs from the same platform. The Pearson correlation coefficient (PCC) was calculated between the reference and the MPEA data and was used as a measure to determine the effects on the performance of the assay by changing one of the above-mentioned parameters.

First, we evaluated the best orientation of the capture probes, which can be attached at either the 3'- or the 5'-end. The original RAKE assay was described on an array platform where the 5'-ends of the oligonucleotides were attached to the surface [17]. Such an arrangement is prone to selfelongation, so an Exonuclease I digest

**FIGURE 2**

Principle of the MPEA. The hybridized sncRNA functions as a primer for an enzymatic elongation in which biotinylated nucleotides are incorporated.

had to be included in the protocol to remove all single stranded capture probes without hybridized target. However, as the Geniom technology allows the synthesis of oligonucleotide capture probes immobilized on either the 5'-end or the 3'-end, both variants were tested. Data from arrays with their 3'-end attached to the surface was of a better specificity (data not shown) and also facilitated a faster protocol as no *ExoI* digest was required, because the free 5'-end cannot serve as a substrate for polymerase extension. Therefore, all further results were obtained from arrays where the 3'-ends of the oligonucleotides were attached to the surface of the chip. The better performance of this modified assay might result from better accessibility of the enzyme and no steric hindrance as the elongation faces the lumen of the microchannel and not the surface.

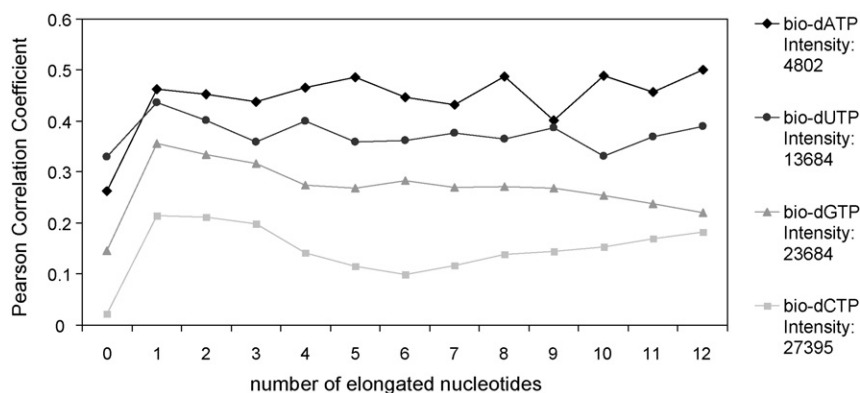
Second, we tested whether an enrichment of sRNAs of 200 nucleotides or less was necessary or whether the MPEA would

work equally well with total RNA. Two micrograms of total RNA and the equivalent of the enriched fraction were analyzed and no difference in the results was observed (data not shown). Therefore the enrichment step was omitted and only total RNA was used, which is a major feature of sample preparation.

Thirdly, the elongation reaction was evaluated. The best nucleotide for the elongation (bio-dATP, bio-dCTP, bio-dGTP and bio-dUTP), the optimal number of nucleotides elongated (0–12) and the concentration of the biotinylated nucleotide (0.4, 4 and 40  $\mu\text{M}$ ) were investigated. The results are shown in Figs 3,4. The specificity as measured by the PCC varied with the nucleotide used for elongation and was significantly lower when biotinylated dGTP or dCTP was used compared with dATP and dUTP. The highest specificity was obtained when using bio-dATP. Interestingly, this was also reflected in the overall array intensity: the higher the specificity, the lower the overall array intensity, measured as fluorescence counts per second. Nearly every feature in the arrays where bio-dCTP and bio-dGTP were used in the elongation reaction showed high intensity, a very improbable situation as this would indicate that every miRNA was expressed. This is not the case. miRNAs were frequently expressed in a highly tissue-specific manner. The intensities with bio-dUTP and especially bio-dATP were significantly lower. From the three different bio-dATP concentrations tested for the elongation (0.4, 4 and 40  $\mu\text{M}$ ), 4  $\mu\text{M}$  gave the most specific signal (Fig. 3) and was chosen for all further assays. To test for the nucleotide incorporation with the best signal-to-noise ratio we designed probes with 0–12 thymidine units at the terminal 5'-end. Figs 2,3 show that the elongation of five nucleotides gave the best performance as measured by the PCC. Most probably steric hindrance is the reason why incorporation of more than five nucleotides does not result in higher signal intensities.

#### Determination of the amount of RNA needed

Previously published data with the RAKE assay was obtained from as much as 20  $\mu\text{g}$  total RNA. Such large sample amounts render the assay inaccessible for small tissue sizes, for example, biopsies. Owing to the small sample volume needed for microfluidic microarrays, it was assumed that significantly lower amounts should be sufficient for the MPEA: the channel volume is 3  $\mu\text{l}$  and the volume used for hybridization is 18  $\mu\text{l}$ . A variety of different amounts of

**FIGURE 3**

Correlation between data from a conventional hybridization assay and the MPEA on the type of biotinylated nucleotides used for elongation ( $\blacklozenge$  = bio-dATP,  $\bullet$  = bio-dUTP,  $\blacktriangle$  = bio-dGTP,  $\blacksquare$  = bio-dCTP) and the number of elongated nucleotides.

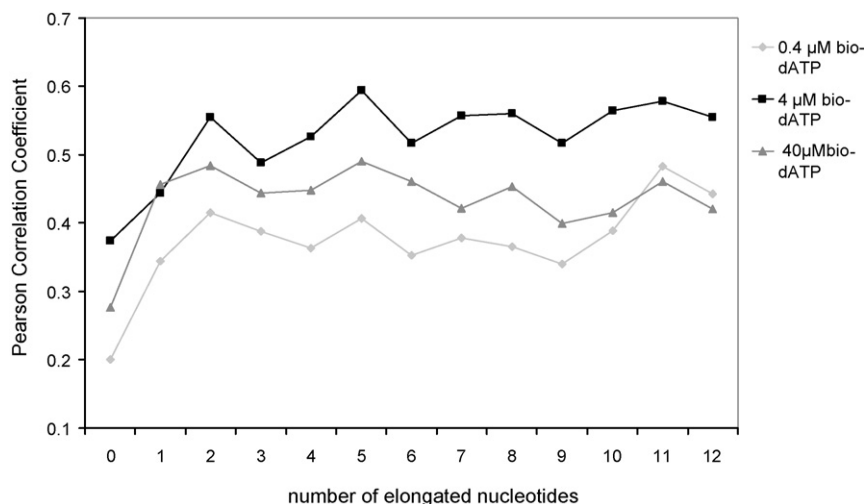


FIGURE 4

Dependency of the performance of the MPEA on the concentration of bio-dATP used ( $\diamond = 0.4 \mu\text{M}$ ,  $\blacksquare = 4 \mu\text{M}$ ,  $\blacktriangle = 40 \mu\text{M}$ ) and the number of nucleotides available for elongation as measured by the Pearson correlation coefficient.

total RNA ranging from 5 to 20 ng was tested and the results are shown in Fig. 5. The quality of the data measured as the correlation with a standard hybridization assay remained nearly constant down to as little as 50 ng and only then was a drop in performance observable. However, even 20 ng of total RNA gave meaningful data. The signals of weakly expressed miRNAs fell into the background, but the moderately to strongly expressed signals were clearly detectable. Thus, experiments with 20 ng still generated informative biological data. These findings indicate that the MPEA is more sensitive than the RAKE assay and can reliably be used with sample amounts in the low ng range.

#### Demonstration of the enhanced specificity of the MPEA

An ability to discriminate especially between closely related members of a miRNA family is crucial for the quality of the results, because different members of a family can fulfill different biological functions [18,19]. The best example of a complex family is provided by the human let-7 family as shown in Fig. 6. Members of this family vary by as little as one nucleotide and the mismatches

frequently lie close to the ends of the molecule (*hsa-let-7b* and *c*, e.g.). To demonstrate the discriminatory power of the MPEA, synthetic RNA-oligonucleotides for each of the eight members of the family were hybridized at 100 pM on individual arrays representing probes for all eight family members. The capture probes for each of the eight *hsa-let-7* family member were present as 100 intra-array replicates on each of the individual arrays. Signal intensities for each *hsa-let-7* member were calculated as the median of the intra-array replicates. The background, calculated from all blank-control features, was subtracted from the signal intensities. For comparison, the highest value, from the capture probes specific for the hybridized RNA-oligonucleotide, was set to '1' and all others were set in relation to this to give a measure for the relative level of cross-hybridization. This assay was performed with both a standard hybridization microarray, where the RNA-oligonucleotides were labeled before the hybridization, and with the MPEA to allow a direct comparison in performance. The results are shown in Fig. 7a,b: each cluster of eight bars is the result of one individual array on which one RNA-oligonucleotide-specific for

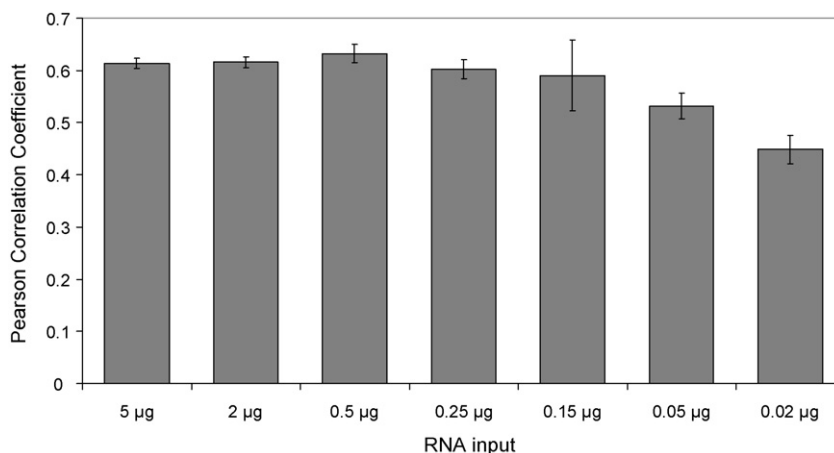


FIGURE 5

Correlation of the MPEA performance to the amount of input total RNA.

U	G	A	G	G	U	A	G	U	A	G	G	U	U	G	U	A	U	A	G	U	U	hsa-let-7a
U	G	A	G	G	U	A	G	U	A	G	G	U	U	G	U	G	U	G	G	U	U	hsa-let-7b
U	G	A	G	G	U	A	G	U	A	G	G	U	U	G	U	A	U	G	G	U	U	hsa-let-7c
A	G	A	G	G	U	A	G	U	A	G	G	U	U	G	C	A	U	A	G	U		hsa-let-7d
U	G	A	G	G	U	A	G	G	A	G	G	U	U	G	U	A	U	A	G	U		hsa-let-7e
U	G	A	G	G	U	A	G	U	A	G	A	U	U	G	U	A	U	A	G	U	U	hsa-let-7f
U	G	A	G	G	U	A	G	U	A	G	U	U	U	G	U	A	C	A	G	U		hsa-let-7g
U	G	A	G	G	U	A	G	U	A	G	U	U	U	G	U	G	C	U	G	U		hsa-let-7i

FIGURE 6

Alignment of the sequence homologs of the human let-7 family. Identical nucleotides are shaded in grey, mismatches in white.

one human let-7 family member was hybridized, as indicated on the x-axis. Each such cluster shows the level of hybridization of the particular *hsa-let-7* member plus its cross-hybridization to capture probes for the other seven members. Although the standard hybridization assay already shows very good discriminatory power even down to single base resolution, there is significantly less cross-hybridization in the MPEA. The reason for this observation is that the MPEA combines the discriminatory power of the standard hybridization with an enzymatic elongation requiring

nearly perfect matches at the 3'-end. Thus the MPEA provides additional specificity particularly at the otherwise difficult-to-discriminate 3'-end.

To express this increased specificity numerically, the mean level of cross-hybridizations for a given member of the human let-7 family was calculated for both assays and the results are shown in Fig. 8. The median levels of cross-hybridization ranged from 2% to 25% for the standard hybridization assay and from 0% to 7% for the MPEA. This again shows that the MPEA significantly increases specificity.

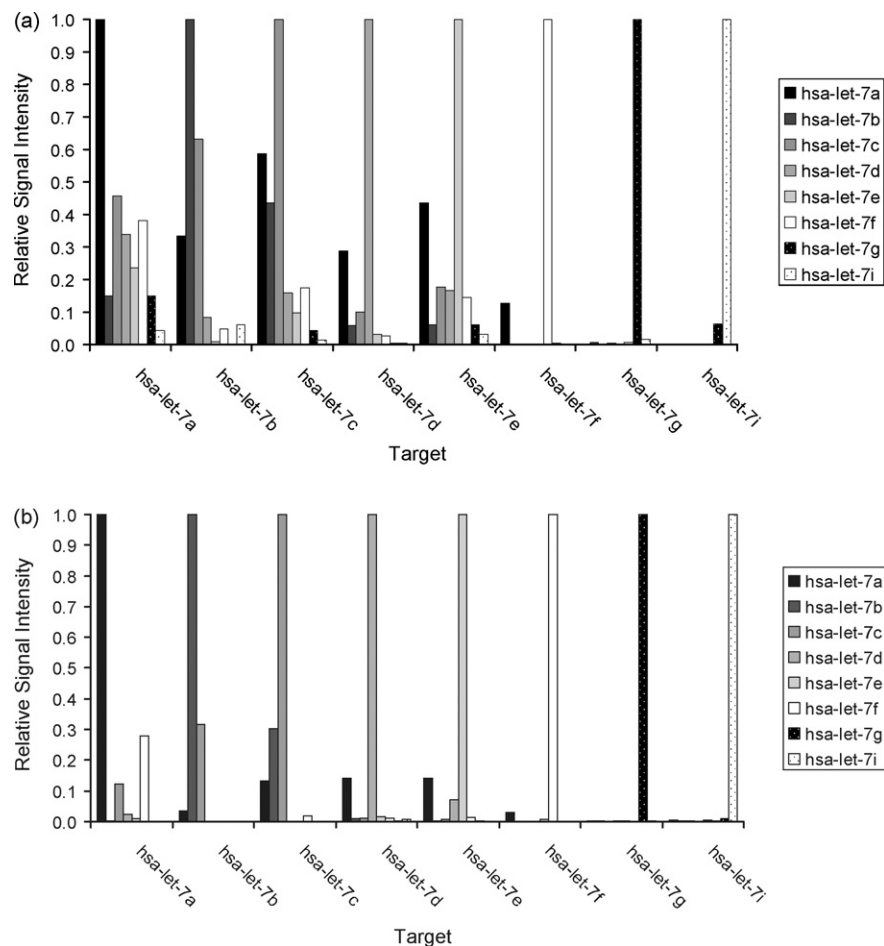
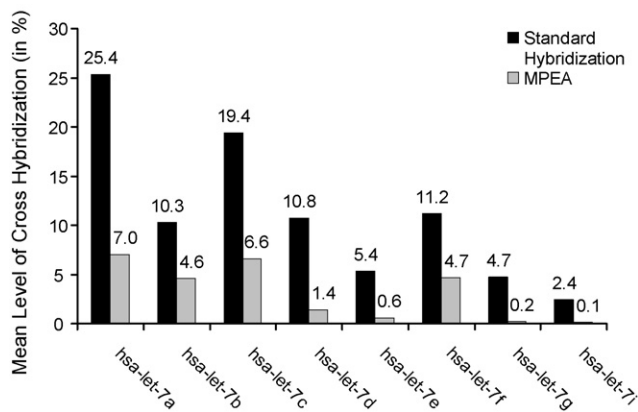


FIGURE 7

Level of cross-hybridization between individual members of the human let-7 family (a) in a conventional microarray and (b) with the MPEA. Clusters on the x-axis indicate the hybridized synthetic RNA-oligonucleotide; the bars represent the relative amount of signal for each member of the family.



**FIGURE 8**  
Mean level of cross-hybridization within the human let-7 family. Black bars: standard hybridization microarray, grey bars: MPEA.

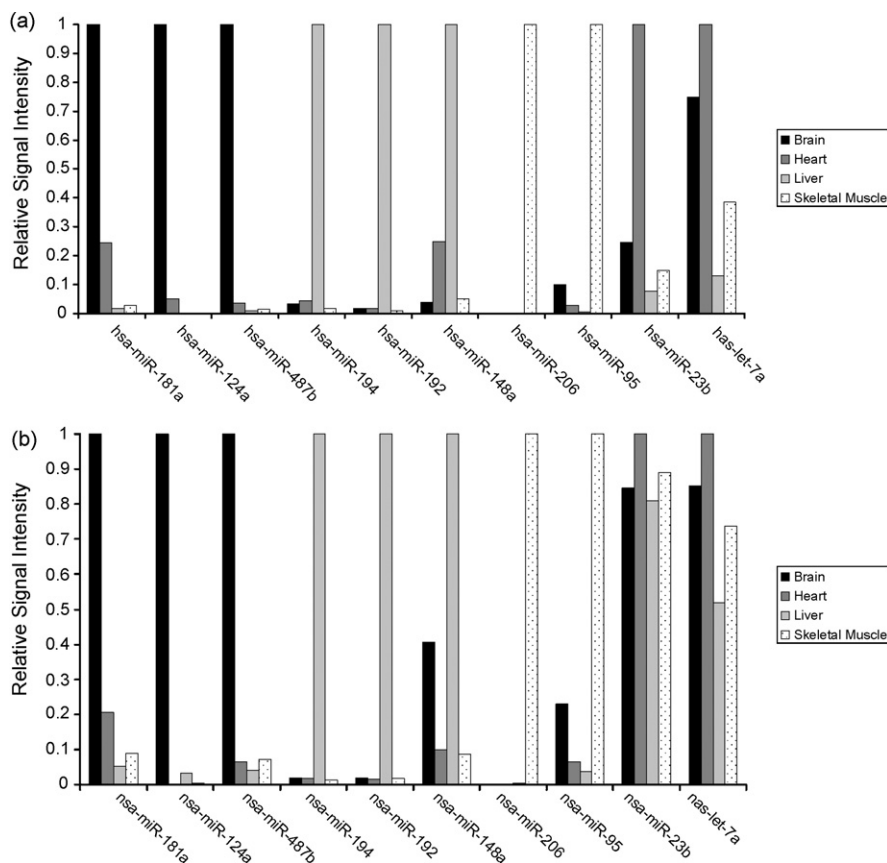
*Correlation with TaqMan real-time PCR assays*

The biological relevance of the MPEA data was assessed by comparing it to results obtained from TaqMan assays, the ‘gold standard’ for miRNA analysis. Commercially available TaqMan probes are frequently designed on outdated sequence information, so only those assays were chosen which reflect the most recent miRBase release and correspond to the capture probes used for the array-based assays. Ten miRNAs were chosen and analyzed for their expression in human brain, heart, liver and skeletal muscle. Nine of these miRNAs show a relatively tissue-specific expression and one has a

broader expression in all four tissues. The expression of a given miRNA in the tissue with the highest intensity was set to ‘1’ and the expression in the other three tissues was set relative to it. The results from the TaqMan and the MPEA are shown in Fig. 9a,b, respectively. It can be clearly seen that all analyzed miRNAs give a similar expression pattern in both assays, indicating the data generated with the MPEA is biologically relevant. However, some differences between the two techniques exist, most markedly seen with *hsa-miR-148a* and *hsa-miR-23b*. We can only hypothesize as to their reason. The action of Dicer generates miRNAs of variable lengths [13] with the most prominent variations at the 3’-end. Therefore, miRNA analysis automatically implicates analysis of mixed populations of miRNAs. Most probably, different detection techniques will resolve this variation with subtle differences.

**Discussion**

The field of sncRNA research is growing rapidly, with new sequences constantly being published. This makes it necessary to have high-throughput tools available which are flexible enough to incorporate any new sequence information without inconvenient and costly set-up fees. Secondly, some sncRNAs, such as the miRNAs, exist in families in which members differ in as little as one nucleotide. Therefore, all analysis tools must be able to detect a single mismatch, even if it is at the end of the molecule. Further, the ideal tool will be sensitive enough to work without any amplification step, because every manipulation has the potential to introduce a bias which may produce misleading results.



**FIGURE 9**  
Comparison of the relative expression level of selected miRNAs in four different human tissues (brain, heart, liver and skeletal muscle), detected with (a) TaqMan assays and (b) MPEA. The strongest signal for a given miRNA was set to 1 and all others were adjusted accordingly.

Microarrays are a convenient tool for the high-throughput analysis, but most commercially available platforms lack flexibility and thus only offer out-dated content. The latter is also the case for many commercially available real-time PCR solutions. Additionally, any microarray technology is based on a conventional hybridization which is strong in detecting mismatches at the central position, but has limitations toward the 3'- and 5'-ends. The RAKE assay as described by [17] provides a solution at least for the 3'-end; the enzymatic step ensures that elongation and thus labeling of the sncRNA only occurs if there is complementarity at the 3'-end. The drawback of this technology is the large amount of input RNA needed (up to 20 µg) and which is not available for a broad range of samples. The recently published adaptation of the RAKE to the BeadArray™ format [23] needs less material, but requires a PCR-based amplification. This shows that several useful approaches are available, but all have their drawbacks.

Here we present a novel assay which combines flexibility, specificity and sensitivity. The febit Geniom microarray technology provides maximum flexibility, as every array can be designed individually within minutes, effortless and without extra cost. This means, for example, that the same day a new miRBase version is released, new information can be incorporated into a microarray. In addition, the MPEA offers two levels of specificity and a selective depletion of unwanted RNA molecules before labeling. During the hybridization step the sequence-specific base-pairing provides a strong discriminatory power in the central position. Then all unbound molecules are depleted by washing the arrays, hence selecting for the correct RNA molecules. During the last step, the enzymatic on-chip labeling, an additional layer of specificity is added at the 3'-end. Altogether, only molecules that pass

both the hybridization and the enzymatic selection steps will be labeled, leading to a significant reduction of background signal compared to other methods. An advantage of MPEA over RAKE is the use of the microfluidic channels which dramatically reduces the amount of sample needed. It provides a sensitivity high enough to enable working with low ng amounts of total RNA without the need for an extra amplification step. This means that even hard-to-obtain samples such as material from fine needle aspiration or LCMs becomes accessible for sncRNA profiling. LCM might prove particularly useful, because it is known that miRNAs are expressed not only in a highly tissue-specific, but also a cell-specific manner [28]. This is likely to hold true for other sncRNAs as well. The standard tissue sample however consists of different cell types, each with a different function and sncRNA expression pattern. If this mixture is analyzed, cell-specific signals might be too dilute to be detectable. Hence, obtaining expression profiles with a high spatial resolution is likely to reveal new and more relevant insights into biological processes.

As a next stage in development, we envisage automating and integrating the MPEA into a single benchtop instrument. This automated microarray processing and detection platform would integrate not only the washing and detection step, but also the on-chip labeling, thus providing researchers with a convenient solution which dramatically reduces hands on time.

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