

Label-free high-throughput microRNA expression profiling from total RNA

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Received May 5, 2011; Revised August 18, 2011; Accepted September 6, 2011

ABSTRACT

MicroRNAs (miRNAs) are key biological regulators and promising disease markers whose detection technologies hold great potentials in advancing fundamental research and medical diagnostics. Currently, miRNAs in biological samples have to be labeled before being applied to most high-throughput assays. Although effective, these labeling-based approaches are usually labor-intensive, time-consuming and liable to bias. Besides, the cross-hybridization of co-existing miRNA precursors (pre-miRNAs) is not adequately addressed in most assays that use total RNA as input. Here, we present a hybridization-triggered fluorescence strategy for label-free, microarray-based high-throughput miRNA expression profiling. The total RNA is directly applied to the microarray with a short fluorophore-linked oligonucleotide Universal Tag which can be selectively captured by the target-bound probes via base-stacking effects. This Stacking-Hybridized Universal Tag (SHUT) assay has been successfully used to analyze as little as 100 ng total RNA from human tissues, and found to be highly specific to homogenous miRNAs. Superb discrimination toward single-base mismatch at the 5' or 3' end has been demonstrated. Importantly, the pre-miRNAs generated negligible signals, validating the direct use of total RNA.

INTRODUCTION

The 1993 discovery of lin-4 in *Caenorhabditis elegans* is the first in a class of endogenous small regulatory RNAs (typical length ~22 nt) later bearing the name microRNA (miRNA) (1). Since then, the total number

of distinct mature miRNAs has grown to more than 17 000 in over 140 species, according to miRBase release 16 (2). As negative post-transcriptional regulators of gene expression (3,4), these tiny molecules play pivotal roles in many fundamental aspects of life, such as plant and animal development, tissue differentiation, metabolic modulation and cell proliferation control (5,6). To appreciate the profound impact that miRNA networks have on our health, one may just consult the simple estimation that at least one-third of all human genes are subject to miRNA regulation (7,8). Indeed, mounting studies have revealed miRNAs as signatures associated with various human diseases (9–11). Recent findings that miRNAs are present in both plasma and saliva in a remarkably stable form brought the anticipated 'miRNA disease markers' even closer (12,13). In the years to come, the pursuits of further elucidating or exploiting miRNAs will continue to intensify among researchers and companies from diverse fields.

This bright future, however, would be unimaginable without the aid of state-of-the-art technology to acquire genome-wide high-quality miRNA expression data, especially when it comes to the intricate nature of miRNAs like small size, wide range of melting temperature and large number of highly homologous sequence variants (14,15). At present, quantitative polymerase chain reaction (qPCR), oligo microarray and deep sequencing are three primary technical platforms for miRNA expression profiling, each with its own strengths and shortcomings (15). Sequencing has the highest discerning power and uncontested advantage of identifying unknown miRNAs; qPCR covers a larger dynamic range and is regularly deemed as the 'gold standard' for gene expression. Nevertheless, the enzyme-based ligation and amplification steps involved in these two methods introduce inherent biases. In addition, long runtime and complicated bioinformatics for data interpretation are extra burdens of sequencing, while the typical throughput of qPCR is

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lagging behind the fast-growing miRNA entries. Generally, sequencing is considered the best choice for discovery of novel miRNAs and analysis of tiny variations, and qPCR the most reliable validation tool of profiling results. Between them, multiple microarray platforms serve as efficient and economic workhorses for vast high-throughput miRNA profiling tasks (16,17). Advantages of microarray also include skip of amplification and modest runtime. Several studies aiming to customize microarrays for improved miRNA profiling performance have been reported in recent years (18–22). However, microarrays are by no means acting perfectly. A major concern is the drawbacks associated with the labeling procedure (15,23), which is essential to current miRNA microarrays. In a recent systematic survey of mainstream commercial miRNA microarrays, all six investigated platforms are label-based (24). Labeling procedures are usually laborious, expensive, some are not suitable for certain type of miRNAs and, most importantly, they are prone to biases, artificial errors and responsible for much of cross-platform variability (16). In fact, current commercial microRNA microarray systems were reported to have poor performances in terms of inter-platform concordance (25). Although some of these problems were partially solved in the RAKE assay devised by Nelson *et al.* (20) via post-hybridization labeling, remaining difficulties accompany the usage of two different enzymes could not be eradicated.

On the other hand, novel efforts have been continuously made to develop alternative non-labeling methods for direct miRNA detection in the past decade (26,27). Proposed approaches include molecular beacon (28), electrochemical device (29), enzyme-based colorimetric detection (30), surface plasmon resonance (SPR) (31), surface-enhanced Raman spectroscopy (SERS) (32), silicon nanowire (33), nanomechanics (34) and so on. Apparently, the label-free approach has won much favor among a wide array of researches aiming to provide innovative solutions for miRNA detection. Unfortunately, even with the usually inadequately addressed specificity issue put aside, most of these techniques came at the price of poor multiplexity, or declined sensitivity, or lack of robustness or dependence on less-accessible sophisticated instruments, and eventually failed to raise significant challenges to established platforms when entering the practical arena. Surprisingly, none of these attempts sought the possibility of reconfiguring the canonical fluorescent microarray, one of the most reliable and widely used expression profiling technologies, to work in a label-free manner.

Here, we report a fluorescent microarray-based label-free method that we termed Stacking-Hybridized Universal Tag (SHUT) assay for simple, robust and accurate high-throughput miRNA profiling. ‘Stacking hybridization’ is referred to the additional stability associated with DNA hybridization reactions wherein two or more DNA oligonucleotides hybridize in a contiguous tandem arrangement to a longer complementary DNA single strand. Mirzabekov’s laboratory first developed the stacking hybridization technology for DNA sequencing on gel-based microarrays (35), and assessed

the stacking effect by measuring the increase of melting temperature and free energy of the duplexes formed by stacking hybridization (36). It has been demonstrated capable of single-nucleotide polymorphism (SNP) detection (37).

Evolved from conventional fluorescent microarray technology, the SHUT assay bears inherent gifts such as excellent multiplexity and high sensitivity, which are still the quests of many other label-free approaches. Furthermore, the SHUT assay allows direct use of total RNA extracted from biological samples as input material, while most established microarray methods may suggest otherwise.

MATERIALS AND METHODS

DNA and RNA sources

Probe DNA oligonucleotides complementary to publicly available miRNA target sequences, Cy3-labeled universal tag (UT) DNA oligonucleotides, PCR primers and RT primers were synthesized and purified by Bioneer (Shanghai) Co., Ltd (Korea). Synthetic RNA were ordered from TaKaRa Biotechnology (Dalian) Co., Ltd (Japan). Total RNA from six human tissues (brain, heart, liver, placenta, skeletal muscle, and thymus) were purchased from Ambion, Inc. (USA) and were used without further fractionation or enrichment.

Microarray fabrication

C6 amine end-linked DNA oligonucleotide probes were dissolved in spotting buffer [pH 9.0, 0.1 M sodium carbonate, 1.5 M betaine, 20% dimethyl sulfoxide (DMSO)] to prepare 20 μ M working solutions. Except the ‘targeting probes’, three negative control probes were included to estimate fluorescence background and background variance; and one (pos-RNA, in specificity and sensitivity experiments) or three (Zip-5, Zip-13 and Zip-21 in tissue miRNA profiling experiments) reference probe(s) were used as exogenous controls. Probes were printed by a commercial arrayer (PersonalArrayer 16, CapitalBio Co., China) to home-made aldehyde-activated glass slides at 24–26°C along with 50–55% humidity. Each slide may contain up to eight individual microarrays. Each probe was spotted in triplicate within an array. Typically, the spot diameter was \sim 150 μ m and the center-to-center spacing was \sim 350 μ m. The spotted slides were incubated in a humid chamber overnight at room temperature, then washed twice for 10 min in 0.1% sodium dodecyl sulfate (SDS), followed by thorough washing with ultrapure water (Milli-Q Synthesis, Millipore, USA). The targeting probe sequences used in specificity and sensitivity experiments, and the sequences of control probes are listed in Supplementary Table S3.

SHUT assay protocol

For miRNA profiling, 2.0 μ g, 0.5 μ g or 0.1 μ g total RNA from each human tissue and 200 nM (final concentration) Cy3-labeled reporter molecule (Universal Tag, or UT, see Supplementary Table S3) were dissolved in hybridization

buffer ($5\times$ SSC, 0.2% SDS, and $0.1\ \mu\text{g}/\mu\text{l}$ random 25-mer DNA). The final mixture volume was $45\ \mu\text{l}$ for each sample, and was heated for 5 min at 90°C and then cooled on ice immediately before assay. Each $45\ \mu\text{l}$ sample solution was dispensed to one well of the SureHyb gasket slide (G2534-60014, Agilent Technologies, Inc., USA), then an array slide was placed down onto the SureHyb gasket slide with the array side facing the target samples. The array/backing slide sandwich was clamped into the SureHyb Hybridization Chamber (Agilent G2534A), and hybridized at 42°C for 16 h in a hybridization oven (Agilent 2545A) with a constant rotation speed of 15 r.p.m. After hybridization, slides were washed in $5\times$ SSC and 0.1% SDS at 30°C for 6 min, and then washed for 3 min twice at room temperature in $0.2\times$ SSC. The slides were immediately dried on a slide centrifuge. For the sensitivity and specificity experiments, the assay process was similar to that of the miRNA profiling, while synthetic miRNAs at various assigned concentrations were used as input sample instead of tissue total RNAs.

Image scanning and data analysis

After hybridization and washing, slides were scanned using a LuxScan 10K Microarray Scanner (CapitalBio Co., China) at constant power and PMT gain settings through a single-color channel (532 nm wavelength). Nonhybridized and artifact-associated spots were removed by both visual inspection and software-guided flags. The raw pixel intensities were extracted using the LuxScan 3.0 software (CapitalBio Co., China). Cy3 median pixel intensity values were background-subtracted. Statistical analysis was performed using OriginPro 8.1 software (OriginLab Co., USA) and R

statistical computing framework 2.12 (<http://www.R-project.org>) (R Development Core Team 2010). Briefly, background-subtracted data were first filtered using their signal/noise ratio (SNR) values with a threshold of 2 (for an SNR lower than threshold the corresponding feature was flagged 'Not found' by assigning its intensity data to 1). Then, 'within array' normalization was performed for different sub-arrays printed on the same slide, using the geometric mean value of multiple external (spiked-in) controls as the normalizing reference. Next, sample data were corrected by subtraction of the blank data (hybridization buffer containing only UT and no miRNA) to remove probe-associated fluorescence. For replicates of the same biological sample on different microarray slides, 'between arrays' normalization was performed using the quantile normalization technique (38) and the 'normalizeBetweenArrays' function in package 'limma' ver. 2.6.1 (39) of R 2.12. Finally, the arithmetic average of \log_2 -transformed normalized values of the replicates was taken as the target miRNA's expression data.

Quantitative PCR

Please see Supplementary Data for a detailed description of the qPCR procedure.

RESULTS

Microarray design strategy

The basic design of our SHUT probe (Figure 1) is a linear assembly of three functional segments: (i) a 5' poly(A) spacer sequence to extend the remaining part of the probe away from the array's solid surface; (ii) a 3'

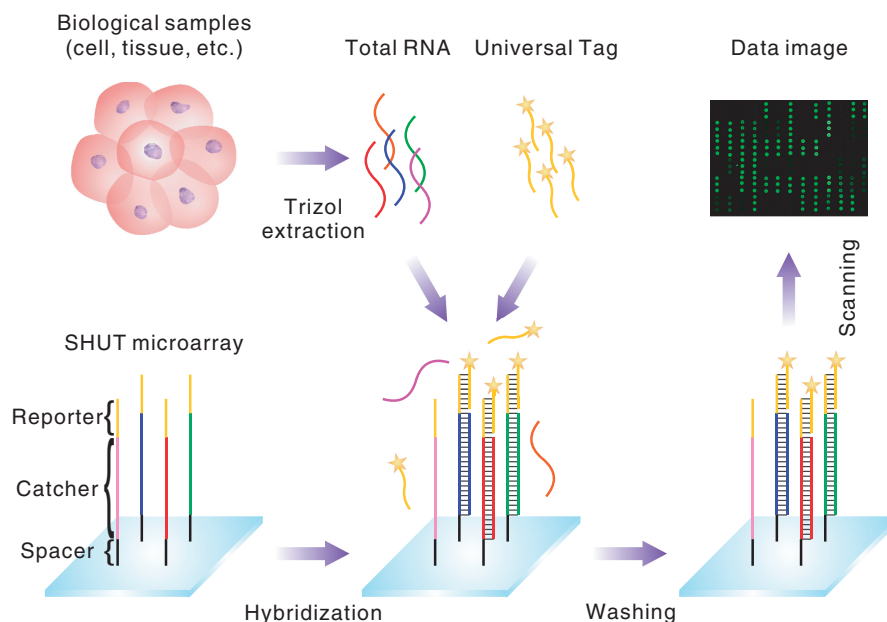


Figure 1. A schematic diagram of the Stacking-Hybridized Universal Tag (SHUT) assay. The sample probe at the top of the figure illustrates the generic structure of the DNA oligonucleotides used on the microarray. The nucleotides at the 5' end of the probe comprise an A_{10} spacer, followed by the variable sequences targeting against specific miRNAs. The 3' end is complementary to the Universal Tag (UT) and shared by all capture probes. A total RNA sample is extracted from cells by Trizol and hybridized to a spotted microarray with the Cy3-linked UT. After hybridization, the slide is washed and scanned by a microarray scanner. The UT specifically bound to spots where probe-miRNA hybridization occurred.

reporter sequence complementary to a fluorophore-conjugated oligomer we termed Universal Tag (UT); and (iii) in between them, a catcher sequence matching a specific miRNA target. The catcher sequences are different while the spacer and reporter are identical for all the probes. According to this design, either the target miRNA or the UT could hybridize to a specific part of the probe; however, only the former (19–23 bp) is thermodynamically favorable under assay condition (42°C). As a result, in the absence of target miRNA, a steady hybridization of the short UT (melting temperature designed below 35°C) to the probe hardly sustain, generating very faint background fluorescence. Conversely, when target miRNA binds to the catcher sequence, the hybridization of the UT to the probe gains extra stability from the base stacking effect between the two closely positioned end nucleotides (one on UT and the other on the miRNA), sufficient to tether the UT to the probe (36). In this way, we combined target capturing and fluorescent signaling in a single step, just like any conventional hybridization assay, but without labeling the targets.

For the whole scheme to work, the starting point is to find a proper oligomer to serve as the Universal Tag. Too short a UT, the duplex could be unlikely to form even if the targets had hybridized to the probes to provide the stacking sites; too long a UT, it could maintain a stable hybridization to the probe without the target miRNAs. This was accounted for experimentally to find an optimal UT length to be used throughout this study. We tested three probe/UT hybrid lengths using a 5' dye-labeled 9-mer DNA oligo (designated Tag-9, see Supplementary Table S3 for its sequence) and three SHUT probes targeting miR-195. The probes (designated P7_miR-195, P8_miR-195 and P9_miR-195, respectively, see Supplementary Table S3 for the sequences) have identical sequences except at the 3' end they are 7 bp, 8 bp and 9 bp complementary to Tag-9, respectively (Figure 2A). miR-195 (200 pM) were used as target. As expected, the target signal intensity (Figure 2B) is positively correlated to the probe/UT hybrid length. The target signal of the 9 bp hybrids is slightly higher than that of the 8 bp hybrids and both significantly exceeds that of the 7 bp hybrids; yet the 9-bp hybrids generates high blank signal while signals of 7 bp and 8 bp hybrids are negligible. Based on the above observations, we designed an 8-mer DNA oligo (one base shorter than Tag-9 at the 5' end) as the Universal Tag (see Supplementary Table S3 for its sequence).

Specificity

A distinctive nature of miRNAs concerning the detection specificity is the presence of highly homologous family members. Many family members differ in only 1–3 nt. This sequence similarity may pose extra difficulties when it is necessary to distinguish specific miRNAs from their family members. Therefore, recognizing such subtle differences is critical for any successful miRNA profiling technology. To examine the specificity of the SHUT assay, 200 pM synthetic human let-7a, let-7b, let-7d and let-7f miRNA target was applied to a microarray containing let-7a, let-7b, let-7d and let-7f probes, respectively. Most

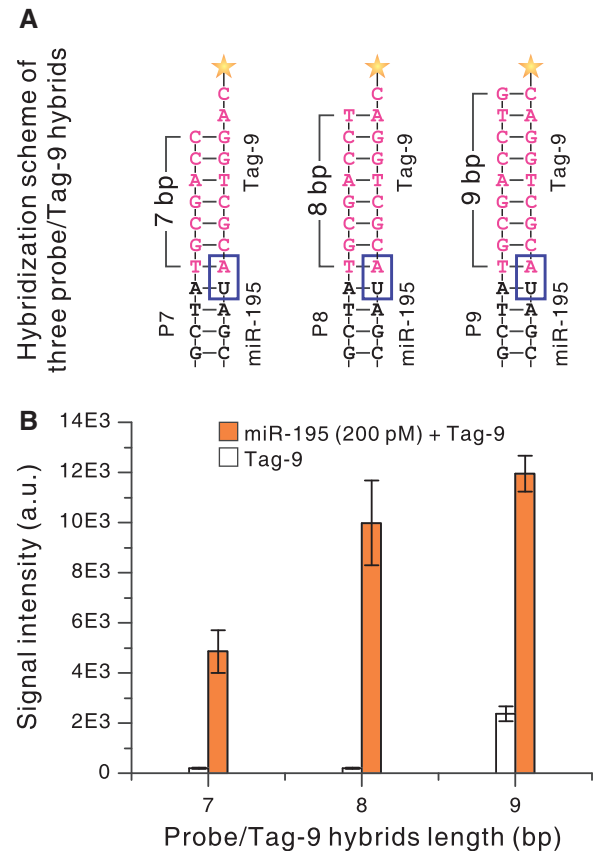


Figure 2. Optimization of Universal Tag (UT) length. (A) Three SHUT probes targeting miR-195 with different hybridization length to a 9-mer fluorescent DNA oligo (Tag-9) were designed (the base-pairing number of the probe/UT complementary duplex was annotated near the probe). The base-stacking site was indicated by a blue box. Note that the remaining part of the probes and miR-195 were omitted. (B) Detection of 200 pM miR-195. Tag-9 concentration: 200 nM. Buffers containing 200 nM Tag-9 without miR-195 served as blank controls. Error bars: SD ($n = 3$).

cross-hybridizations observed were under 25% except for probe let-7f and miRNA let-7a (the difference between let-7a and let-7f is a single-base mismatch in the middle of the sequence), which is well acceptable for the purpose of discrimination (Figure 3A). The few higher cross-hybridization instances (mostly single-nucleotide A/G or C/T substitutions) could be further reduced via probe design optimization by adoption of proposed single-base mismatch discrimination microarray strategies such as ProDeG (40).

In light of previous report by Mirzabekov's group that either a single-base mismatch, overlap or single gap in stacking site would effectively hinder the stacking interaction (36), we expect the SHUT assay to possess extraordinary specificity toward single-base terminal variations. This was first demonstrated by detecting two miRNAs (miR-106a and miR-17) whose only difference is the first nucleotide at the 5' end position. Each of the miRNAs was individually applied to an array containing both of the probes, and comparison was made between the signals generated on the same probes (Figure 3B). In both cases, a sharp signal drop (higher than 90%) was

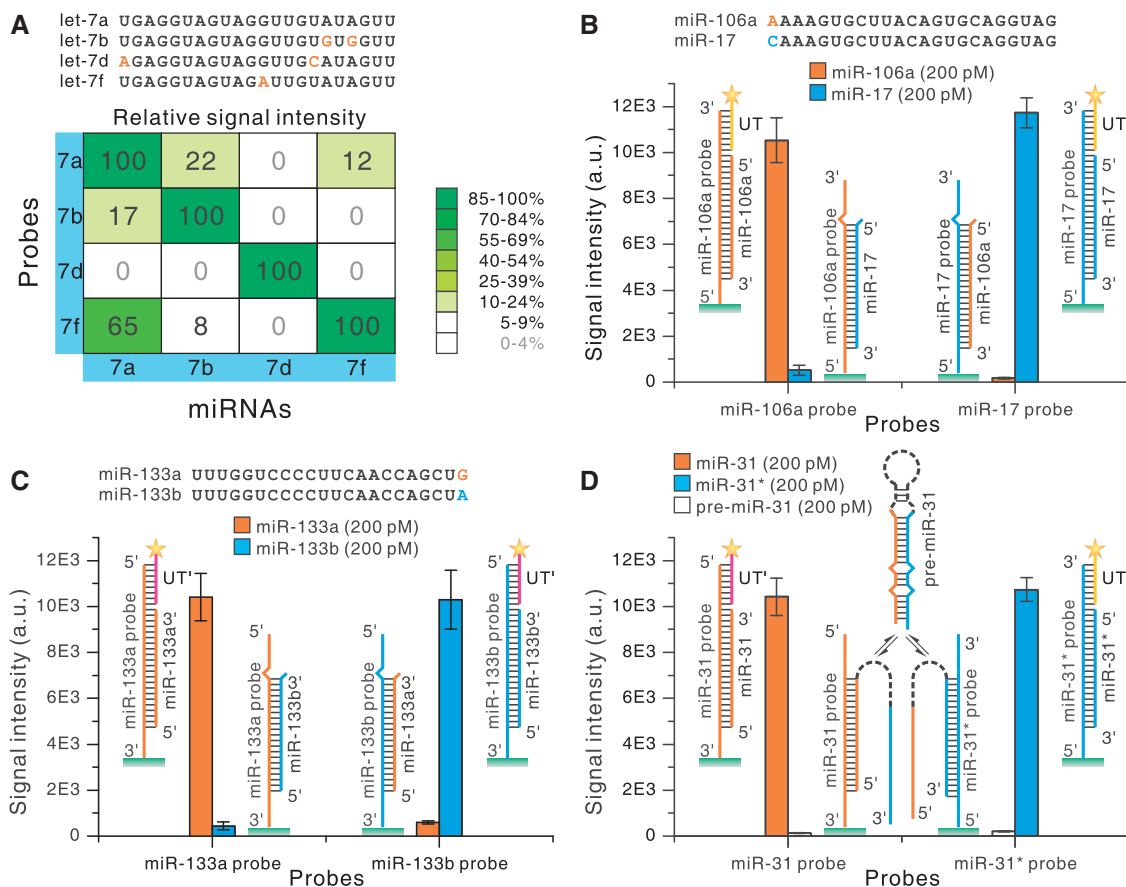


Figure 3. Specificity of the SHUT assay. All error bars are standard deviation of three replicates ($n = 3$). **(A)** Discrimination of let-7 family members. Signals were reported as the relative percentages of the intended target of each probe (taken as 100%). Positions that let-7b, let-7d and let-7f have different bases to let-7a are highlighted. **(B)** Discrimination of miRNAs with single-base difference at the 5' end. Hybridization schemes drawn near each column show typical reaction results of corresponding probe/miRNA/UT combination. Probes are depicted in the same color as its target. UT (yellow) and UT' (magenta) stands for 5' and 3' dye-linked universal tag, respectively. All hybridization duplexes are drawn according to the actual base-pairing numbers. **(C)** Discrimination of miRNAs with single-base difference at the 3' end. **(D)** Pre-mature miRNA discrimination. The stem-loop structure shown in the center is pre-miR-31, the common precursor of two mature human miRNAs: miR-31 (orange) and miR-31* (blue). Dashed line in pre-miR-31 stands for nucleotides not included in any of the two mature miRNA sequences (solid line). All hybridization duplexes are drawn according to the actual base-pairing numbers. Note that under hybridization condition, the precursor is in an equilibrium of stem-loop/free conformation, which is indicated by the double-direction arrows.

observed for the mismatch miRNA. Next, we designed 3'-immobilized probes and 3'-dye universal tag (designated UT', whose sequence is the reverse of the original 5'-dye UT) to detect two miRNAs (miR-133a and miR-133b) only that differs at their 3' end position, and obtained similar results (Figure 3C).

Another potential factor affecting the miRNA assay specificity is the co-existence of pri-miRNAs and pre-miRNAs *in vivo*. These RNA species contain sequences identical to mature miRNAs, yet only the mature ones are the active players that are assembled into RNA-induced silencing complexes (RISCs) (41). Consequently, unless the cross-hybridization of pri- and pre-miRNAs prevented or effectively suppressed, the data obtained from an miRNA assay has to be considered prone to false-positive errors (14). A common practice is to reduce the complexity of biological samples through fractionation to isolate the low-molecular-weight miRNAs from the more abundant high-molecular-weight species before miRNA profiling (19,42,43). However, our

SHUT method can process total RNA directly extracted from biological samples. This is demonstrated by subjecting a synthesized precursor human miRNA, pre-miR-31 (44), along with either of the two mature miRNAs (miR-31 and miR-31*) derived from it to the SHUT assay. The result showed that the precursor's signal is <5% of that of the mature miRNA in both cases (Figure 3D).

Sensitivity and dynamic range

miRNAs represent only a small fraction (~0.01%) of the mass of a total RNA sample (43), yet its typical expression level spans over four orders of magnitude (45). Hence, sensitivity and linear dynamic range are critical determinants when assessing a miRNA profiling method. To measure the dynamic range and limit of detection (LoD) of the SHUT assay, we conducted the hybridization using an equimolar mixture of two synthetic miRNAs (let-7b and miR-195). The concentration series varied from 200 pM down to 20 fM. A short single-strand RNA (pos-RNA)

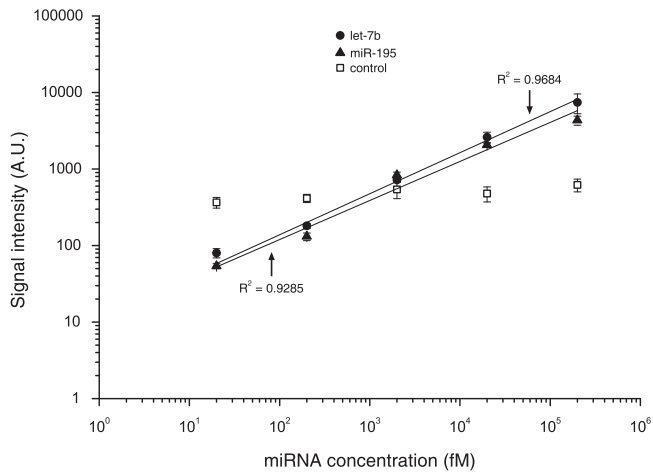


Figure 4. Sensitivity and dynamic range of SHUT assay. Target miRNAs were assayed at concentrations 20 fM, 200 fM, 2 pM, 20 pM and 200 pM, respectively. An external control RNA (pos-RNA) were spiked in at a fixed concentration of 2 pM. Error bars: SD ($n = 3$). The obtained miRNA concentration-signal data were fitted using a power function model and the corresponding COD (correlation of determination) R^2 were indicated by arrows.

at a fixed concentration of 2 pM was spiked-in as an external control. Figure 4 shows that as low as 20 fM (corresponding to 0.9 amol) target miRNA can be detected. The coefficients of determination (CoD $R^2 = 0.968$ for let-7b and 0.928 for miR-195) indicate that the miRNA analysis by SHUT assay can be made with a high degree of confidence across the measured concentration range.

High-throughput miRNA profiling using total RNA from human tissues

The SHUT assay’s applicability for real biological samples was tested by obtaining the expression profiles of a cohort of 121 miRNAs in total RNA extracted from six human tissues (Figure 5). For each tissue, 2.0 μg total RNA was used per assay. As the heat map shows, the measured expression level for some individual miRNAs are stable across different tissues, while for many others it varies in a wide range; consequently, the collective expression of the cohort among different tissues is highly differentiated.

As a preliminary quality assessment for the profiling data, two basic features were examined: (i) Repeatability. The profiling experiments were performed on four replicate microarray slides. Statistical tests showed all data were not significantly drawn from a normally distributed population at $\alpha = 0.05$ level. For each tissue, the mean expression level of all 121 miRNAs from the four micro-arrays showed no significant differences at $\alpha = 0.01$ level (ANOVA test) to each other, indicating a good repeatability. (ii) Quantification capability. The result showed that the signal produced by three spiked-in external control miRNAs well maintained their original concentration ratios (linearity of log signals of the three miRNAs: $R^2 > 0.97$), indicates a good quantification capability.

Further validation of the SHUT assay data was carried by comparison of the expression levels of 12 miRNAs (randomly picked from the 121-plex cohort) among the

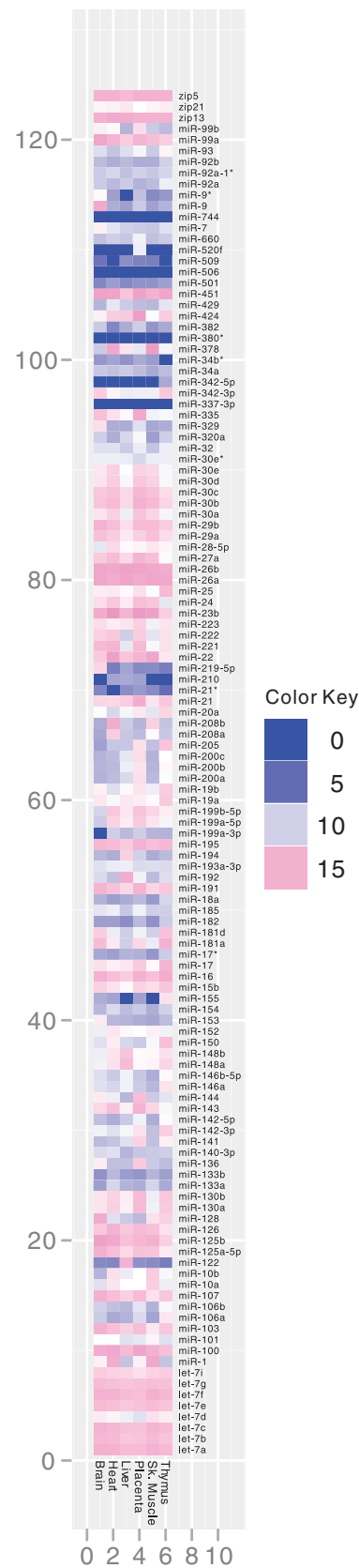


Figure 5. Heat map of expression profile of 121 miRNAs in six human tissues. Color represents log2-transformed expression data. The names of the miRNAs are in accordance with miRBase Release 16. Three non-human miRNAs (Zip-21, Zip-5 and Zip-13) were spiked-in as external control to serve data normalization. The color key indicates the signal intensities based on their log2 values. Error bars: SD ($n = 4$).

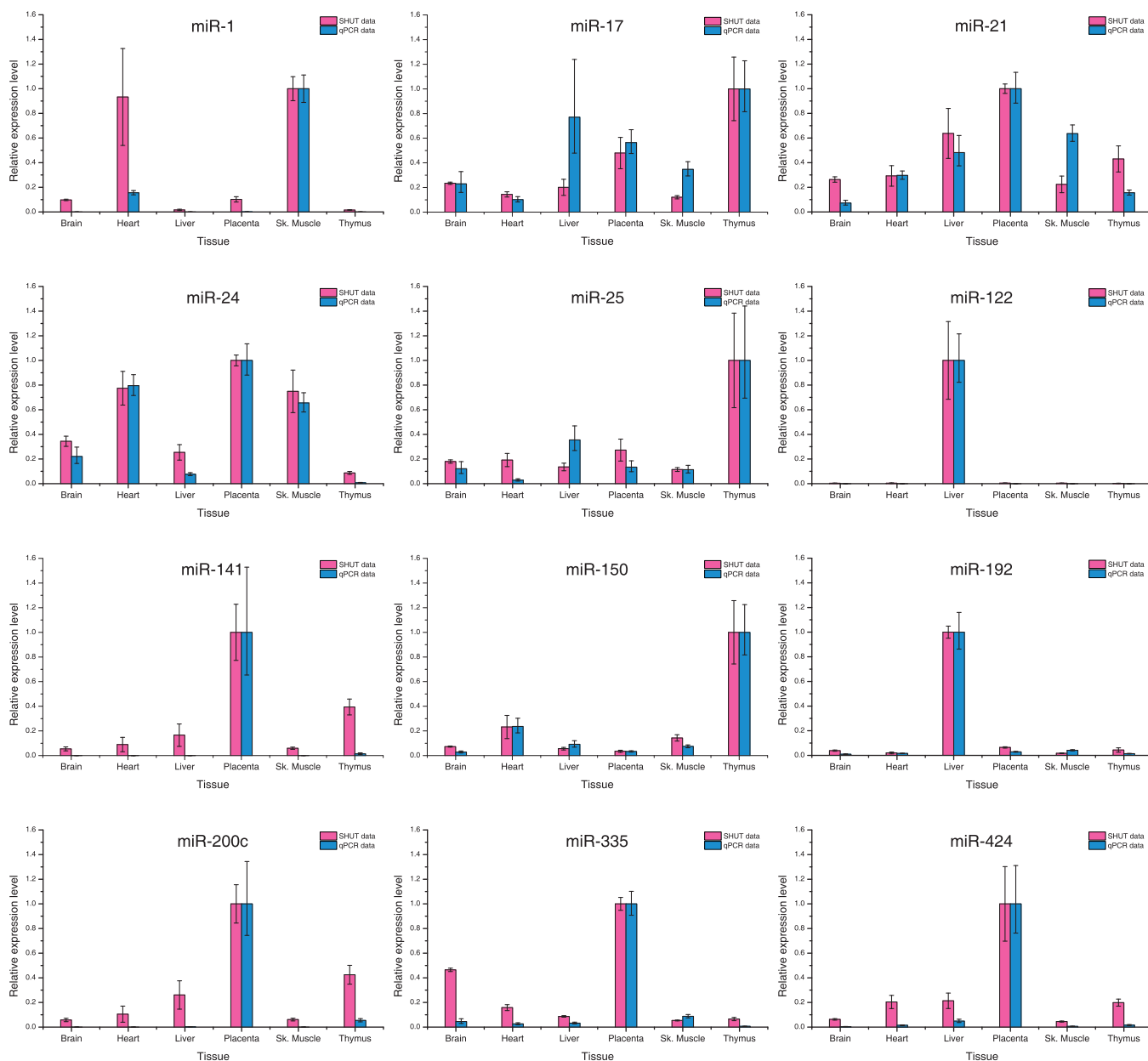


Figure 6. Quantitative real-time PCR (qRT-PCR) verification of SHUT microarray profiling results. Twelve out of the 121 miRNAs that went the high-throughput profiling assay were randomly picked for comparison. Relative expression using either qRT-PCR (azure) or SHUT assay (magenta) were individually plotted for each of these miRNAs, and each miRNA's expression levels in each tissue are reported as the fraction of the expression level in the tissue in which that miRNA is most abundant. Error bars: SD ($n = 3$ for qPCR and $n = 4$ for SHUT assay).

same tissues with their qRT-PCR results (Figure 6). The data from these two sources highly agreed with each other.

Finally, the lowest sample requirement of the SHUT assay was probed. Twenty miRNAs were assayed from 2.0, 0.5 and 0.1 μg total RNA of brain tissue (Figure 7). As we expected, the detected signal level for each individual miRNA fell as the input amount decreased from 2.0 to 0.5 μg , and dropped further while only 100 ng total RNA was used. Nonetheless, the data also suggested that most miRNAs are still detectable by the SHUT microarray assay even when the sample input is extremely low. In addition, more importantly, the profiling results as a whole are consistent for the three different total RNA inputs.

DISCUSSION

As a contradiction to common practice that miRNA samples must be labeled before assay, the SHUT strategy distinguishes itself from conventional methods in that it denies the necessity of labeling procedures. In particular, this strategy is realized in fluorescent microarray format, which is long desired since it may grant label-free approaches the power of high-throughput along with high sensitivity/dynamic range performance. Another fluorescent label-free strategy is molecular beacon, which emits fluorescence upon hybridization of an unlabeled target nucleic acid by separation of the intramolecular-neighbored quencher-fluorophore pair.

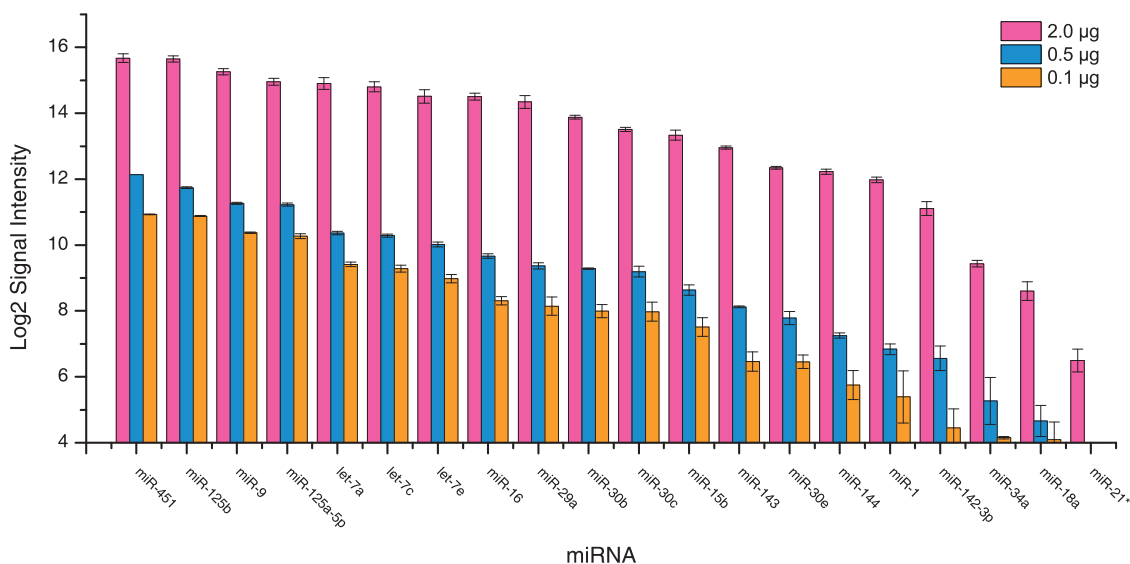


Figure 7. SHUT assay results of 20 miRNAs using different amount (2.0, 0.5, 0.1 µg) of total RNA from human brain tissue as input samples. Error bars: SD ($n = 3$).

However, to employ molecular beacons in the design of microarrays would require extra modification of the microarray probes with a fluorophore and its pairing quencher, which may dramatically increase the cost for high-throughput detection. Not only that, the hairpin structure of the molecular beacon immobilized on glass surface are susceptible to denaturation, which eventually leads to spontaneous departure of the quencher from the fluorophore, generating high background noise (46). As a result, molecular beacon is well accepted for solution-phase detection of nucleic acids, thus excluded from multiplex tasks (27). In contrast, the solid-phase SHUT assay is ideal for high-throughput profiling. It needs no additional modifications of the microarray probes, and efficiently suppresses the background signals by elevating hybridization temperature to 7.6°C higher than the T_m of UT to prevent it from attaching to the free probes.

The SHUT assay is sensitive to both central and terminal differences of miRNAs. Several enzyme-based labeling methods have been reported to be sensitive to 3' end mismatches (20), but they are unable to detect 5' end mismatches due to the 3' activity of the enzymes. Besides, for miRNAs with natural modifications at 3' ends such as plant miRNAs (47), enzymatic labeling would be problematic. Chemical labeling methods are insensitive to the 3' end modifications, but are suspicious of introducing bias by selectively labeling certain nucleotides with higher efficiency than others (23). Being both enzyme- and label-free, the SHUT assay is immune to these drawbacks. Its 5' and 3' end single-mismatch specificity has been well demonstrated in this study. To the best of our knowledge, there is no other array-based miRNA detection approach that can discriminate both 5' and 3' end single-nucleotide alteration. Because of this unique advantage, the SHUT assay could also serve as a valuable tool when miRNA end-sequence variations become a major

concern of investigation (48,49). By expanding probe coverage, the resolution of SHUT assay could be easily tailored to offer more detailed information, such as the expression of miRNA isoforms (isomiRs). Currently, such in-depth assay works are predominately accomplished using sequencing-based approaches (50,51).

The interference caused by precursors is often underestimated, if not overlooked, in most miRNA assay techniques. For assays rely solely on the conformational difference (linear versus hairpin) to tell mature miRNAs from their precursors, it is crucial to sustain the stability of the precursor's stem-loop under assay conditions. However, we have experimentally observed that at 42°C miRNA precursors could produce substantial false-positive signal due to its unfolding and subsequent competitive hybridization to the probes (Supplementary Figure S1, right). Many other assays may suffer from this phenomenon since by their working scheme hybridization means signaling. Yet, this is not the case for our SHUT assay. By simply choosing the other end of target miRNA as the stacking site (Supplementary Figure S1, left) we can perfectly eliminate precursor's interference without impairing the ability to report mature miRNAs. This is a unique benefit of utilizing the base-stacking mechanism for signal transduction. Briefly, if additional nucleotides present at the stacking site (as in the case that pre-miR-31 hybridizes to the miR-31 probe, Supplementary Figure S1, left), the stacking-induced hybridization enhancing effect will diminish as the base-stacking interaction seriously disrupted. In fact, just one extra nucleotide at the miRNA's 'stacking end' would be sufficient to cause a striking 10-fold decrease of signal (see Supplementary Data and Supplementary Figure S2).

The sensitivity of many established miRNA assay protocols might be impaired by factors such as sample loss and inefficient reaction, since one individual or a combination of the following procedures is used in those

assays: (i) RNA size fractionation; (ii) chemical labeling; and (iii) sample purification. In the SHUT assay, sample loss is avoided to a maximum extent by allowing direct use of total RNA without any further manipulations. On the other hand, the excessive concentration of the fluorescent UT used in the hybridization buffer helps to maintain a high signal-transduction ratio for the target-hybridized probes. Taken together, the above factors ensure a high sensitivity performance of the assay.

The complexity of real biological samples may seriously undermine a molecular assay's quantitative precision. Therefore, data validation is our main focus on the 121-plex miRNA profiling from different human tissues. By matching the SHUT profiling data against that from qPCR, we found that these two results correlated well with each other, indicating a reliable performance of the SHUT method under real application conditions. Further experimental inquiry of the lower limit of sample requirement suggests that (i) good data reproducibility as the total RNA input amount decreased to a minimal level and (ii) relatively high sensitivity in the context of using limited raw sample without miRNA enrichment.

For molecular biology assays, any improvement on reagent consumption and operational convenience could be translated to economical efficiency. In SHUT assay, the removal of labeling (fractionation/purification as well) procedure significantly reduces the consumption of expensive chemicals and bench works required in conventional methods. In addition, simplification of handling protocol also helps to minimize potential experimental failures caused by human errors.

In summary, we have designed a hybridization-triggered fluorescence strategy for label-free high-throughput miRNA expression profiling, which is superior to most existing chemical and enzymatic labeling methods in the following aspects: (i) fluorescent signaling without the aid of enzymes; (ii) sharp specificity toward end sequence variations and mature/precursor miRNAs; (iii) excellent sensitivity with a linear dynamic range over four orders of magnitude; and (iv) substantial reduction of expenses on reagents, labor and time. Furthermore, the principle could be flexibly adapted to many existing assay formats (PCR, suspension arrays, etc.). From a methodological perspective, the base-stacking approach described here opens up new opportunities to implement hybridization-triggered fluorescence strategy on solid-phase detection platforms, whereas similar strategies based on molecular beacons are almost exclusively used in solution-phase applications.

SUPPLEMENTARY DATA

Supplementary Data are available at NAR Online.

ACKNOWLEDGEMENTS

We are grateful to Prof. Hongwei Ma for his assistance in microarray printing and scanning. We also thank Prof. Yimin Zhu for discussions.

FUNDING

National Natural Science Foundation of China (No.60701018); and Suzhou Municipal Science and Technology Project (ZXG0711). Funding for open access charge: National Natural Science Foundation of China (No. 60701018).

Conflict of interest statement. None declared.

REFERENCES

- Lee, R.C., Feinbaum, R.L. and Ambros, V. (1993) The *C. elegans* heterochronic gene *lin-4* encodes small RNAs with antisense complementarity to *lin-14*. *Cell*, **75**, 843–854.
- Kozomara, A. and Griffiths-Jones, S. (2011) miRBase: integrating microRNA annotation and deep-sequencing data. *Nucleic Acids Res.*, **39**, D152–D157.
- Wightman, B., Ha, I. and Ruvkun, G. (1993) Posttranscriptional regulation of the heterochronic gene *lin-14* by *lin-4* mediates temporal pattern formation in *C. elegans*. *Cell*, **75**, 855–862.
- Filipowicz, W., Bhattacharyya, S.N. and Sonenberg, N. (2008) Mechanisms of post-transcriptional regulation by microRNAs: are the answers in sight? *Nat. Rev. Genet.*, **9**, 102–114.
- Kato, M. and Slack, F.J. (2008) microRNAs: small molecules with big roles – *C. elegans* to human cancer. *Biol. Cell*, **100**, 71–81.
- Liu, N. and Olson, E.N. (2010) MicroRNA regulatory networks in cardiovascular development. *Dev. Cell*, **18**, 510–525.
- Lewis, B.P., Burge, C.B. and Bartel, D.P. (2005) Conserved seed pairing, often flanked by adenosines, indicates that thousands of human genes are microRNA targets. *Cell*, **120**, 15–20.
- Xie, X.H., Lu, J., Kulbokas, E.J., Golub, T.R., Mootha, V., Lindblad-Toh, K., Lander, E.S. and Kellis, M. (2005) Systematic discovery of regulatory motifs in human promoters and 3' UTRs by comparison of several mammals. *Nature*, **434**, 338–345.
- Volinia, S., Calin, G.A., Liu, C.-G., Ambs, S., Cimmino, A., Petrocca, F., Visone, R., Iorio, M., Roldo, C., Ferracin, M. *et al.* (2006) A microRNA expression signature of human solid tumors defines cancer gene targets. *Proc. Natl Acad. Sci. USA*, **103**, 2257–2261.
- Zhao, Y., Ransom, J.F., Li, A., Vedantham, V., von Drehle, M., Muth, A.N., Tsuchihashi, T., McManus, M.T., Schwartz, R.J. and Srivastava, D. (2007) Dysregulation of cardiogenesis, cardiac conduction, and cell cycle in mice lacking miRNA-1-2. *Cell*, **129**, 303–317.
- Du, C., Liu, C., Kang, J., Zhao, G., Ye, Z., Huang, S., Li, Z., Wu, Z. and Pei, G. (2009) MicroRNA miR-326 regulates TH-17 differentiation and is associated with the pathogenesis of multiple sclerosis. *Nat. Immunol.*, **10**, 1252–1259.
- Mitchell, P.S., Parkin, R.K., Kroh, E.M., Fritz, B.R., Wyman, S.K., Pogosova, A.G., Lippman, E.L., Petroski, A., O'Brian, K.C., Allen, A. *et al.* (2008) Circulating microRNAs as stable blood-based markers for cancer detection. *Proc. Natl Acad. Sci. USA*, **105**, 10513–10518.
- Nikiforov, A.Y., Aspden, L.M., Seethala, R.R. and Mantha, G.S. (2009) MicroRNA expression profiling as a tool for diagnosis of salivary duct tumors. *J. Mol. Diagn.*, **11**, 655–656.
- Cissell, K.A., Shrestha, S. and Deo, S.K. (2007) MicroRNA detection: challenges for the analytical chemist. *Anal. Chem.*, **79**, 4754–4761.
- Baker, M. (2010) MicroRNA profiling: separating signal from noise. *Nat. Meth.*, **7**, 687–692.
- Li, W. and Ruan, K. (2009) MicroRNA detection by microarray. *Anal. Bioanal. Chem.*, **394**, 1117–1124.
- Yin, J.Q., Zhao, R.C. and Morris, K.V. (2008) Profiling microRNA expression with microarrays. *Trends Biotechnol.*, **26**, 70–76.
- Liu, C.-G., Calin, G.A., Meloon, B., Gamlie, N., Sevignani, C., Ferracin, M., Dumitru, C.D., Shimizu, M., Zupo, S., Dono, M. *et al.* (2004) An oligonucleotide microchip for genome-wide microRNA profiling in human and mouse tissues. *Proc. Natl Acad. Sci. USA*, **101**, 9740–9744.

19. Thomson, J.M., Parker, J., Perou, C.M. and Hammond, S.M. (2004) A custom microarray platform for analysis of microRNA gene expression. *Nat. Meth.*, **1**, 47–53.
20. Nelson, P.T., Baldwin, D.A., Scearce, L.M., Oberholtzer, J.C., Tobias, J.W. and Mourelatos, Z. (2004) Microarray-based, high-throughput gene expression profiling of microRNAs. *Nat. Meth.*, **1**, 155–161.
21. Wang, H., Ach, R.A. and Curry, B. (2007) Direct and sensitive miRNA profiling from low-input total RNA. *RNA*, **13**, 151–159.
22. Liang, R.-Q., Li, W., Li, Y., Tan, C.-y., Li, J.-X., Jin, Y.-X. and Ruan, K.-C. (2005) An oligonucleotide microarray for microRNA expression analysis based on labeling RNA with quantum dot and nanogold probe. *Nucleic Acids Res.*, **33**, e17.
23. Wark, A.W., Lee, H.J. and Corn, R.M. (2008) Multiplexed detection methods for profiling microRNA expression in biological samples. *Angew. Chem. Inter. Ed.*, **47**, 644–652.
24. Git, A., Dvinge, H., Salmon-Divon, M., Osborne, M., Kutter, C., Hadfield, J., Bertone, P. and Caldas, C. (2010) Systematic comparison of microarray profiling, real-time PCR, and next-generation sequencing technologies for measuring differential microRNA expression. *RNA*, **16**, 991–1006.
25. Sato, F., Tsuchiya, S., Terasawa, K. and Tsujimoto, G. (2009) Intra-Platform repeatability and inter-platform comparability of microRNA microarray technology. *PLoS ONE*, **4**, e5540.
26. Hunt, E.A., Goulding, A.M. and Deo, S.K. (2009) Direct detection and quantification of microRNAs. *Anal. Biochem.*, **387**, 1–12.
27. Cissell, K. and Deo, S. (2009) Trends in microRNA detection. *Anal. Bioanal. Chem.*, **394**, 1109–1116.
28. Hartig, J.S., Grüne, I., Najafi-Shoushtari, S.H. and Famulok, M. (2003) Sequence-specific detection of microRNAs by signal-amplifying ribozymes. *J. Am. Chem. Soc.*, **126**, 722–723.
29. Pöhlmann, C. and Sprinzl, M. (2010) Electrochemical detection of microRNAs via gap hybridization assay. *Anal. Biochem.*, **82**, 4434–4440.
30. Su, X., Teh, H.F., Lieu, X. and Gao, Z. (2007) Enzyme-based colorimetric detection of nucleic acids using peptide nucleic acid-immobilized microwell plates. *Anal. Biochem.*, **79**, 7192–7197.
31. Fang, S., Lee, H.J., Wark, A.W. and Corn, R.M. (2006) Attomole microarray detection of microRNAs by nanoparticle-amplified SPR imaging measurements of surface polyadenylation reactions. *J. Am. Chem. Soc.*, **128**, 14044–14046.
32. Driskell, J.D. and Tripp, R.A. (2010) Label-free SERS detection of microRNA based on affinity for an unmodified silver nanorod array substrate. *Chem. Commun.*, **46**, 3298–3300.
33. Zhang, G.-J., Chua, J.H., Chee, R.-E., Agarwal, A. and Wong, S.M. (2009) Label-free direct detection of MiRNAs with silicon nanowire biosensors. *Biosens Bioelectron.*, **24**, 2504–2508.
34. Husale, S., Persson, H.H.J. and Sahin, O. (2009) DNA nanomechanics allows direct digital detection of complementary DNA and microRNA targets. *Nature*, **462**, 1075–1078.
35. Parinov, S., Barsky, V., Yershov, G., Kirillov, E., Timofeev, E., Belgovskiy, A. and Mirzabekov, A. (1996) DNA sequencing by hybridization to microchip octa- and p. *Nucleic Acids Res.*, **24**, 2998–3004.
36. Vasiliskov, V.A., Prokopenko, D.V. and Mirzabekov, A.D. (2001) Parallel multiplex thermodynamic analysis of coaxial base stacking in DNA duplexes by oligodeoxyribonucleotide microchips. *Nucleic Acids Res.*, **29**, 2303–2313.
37. Rangel-López, A., Maldonado-Rodríguez, R., Salcedo-Vargas, M., Espinosa-Lara, M., Méndez-Tenorio, A. and Beattie, K. (2005) Low density DNA microarray for detection of most frequent TP53 missense point mutations. *BMC Biotechnol.*, **5**
38. Bolstad, B.M., Irizarry, R.A., Astrand, M. and Speed, T.P. (2003) A comparison of normalization methods for high density oligonucleotide array data based on variance and bias. *Bioinformatics*, **19**, 185–193.
39. Smyth, G.K. and Speed, T. (2003) Normalization of cDNA microarray data. *Methods*, **31**, 265–273.
40. Lee, I., Ajay, S.S., Chen, H., Maruyama, A., Wang, N., McInnis, M.G. and Athey, B.D. (2008) Discriminating single-base difference miRNA expressions using microarray Probe Design Guru (ProDeG). *Nucleic Acids Res.*, **36**, e27.
41. Hutvagner, G. and Zamore, P.D. (2002) A microRNA in a multiple-turnover RNAi enzyme complex. *Science*, **297**, 2056–2060.
42. Lu, J., Getz, G., Miska, E.A., Alvarez-Saavedra, E., Lamb, J., Peck, D., Sweet-Cordero, A., Ebert, B.L., Mak, R.H., Ferrando, A.A. et al. (2005) MicroRNA expression profiles classify human cancers. *Nature*, **435**, 834–838.
43. Shingara, J., Keiger, K., Shelton, J., Laosinchai-Wolf, W., Powers, P., Conrad, R., Brown, D. and Labourier, E. (2005) An optimized isolation and labeling platform for accurate microRNA expression profiling. *RNA*, **11**, 1461–1470.
44. Lund, E., Güttinger, S., Calado, A., Dahlberg, J.E. and Kutay, U. (2004) Nuclear export of microRNA precursors. *Science*, **303**, 95–98.
45. Cummins, J.M., He, Y., Leary, R.J., Pagliarini, R., Diaz, L.A., Sjoblom, T., Barad, O., Bentwich, Z., Szafrańska, A.E., Labourier, E. et al. (2006) The colorectal microRNAome. *Proc. Natl Acad. Sci. USA*, **103**, 3687–3692.
46. Wang, H., Li, J., Liu, H., Liu, Q., Mei, Q., Wang, Y., Zhu, J., He, N. and Lu, Z. (2002) Label-free hybridization detection of a single nucleotide mismatch by immobilization of molecular beacons on an agarose film. *Nucleic Acids Res.*, **30**, e61.
47. Yu, B., Yang, Z.Y., Li, J.J., Minakhina, S., Yang, M.C., Padgett, R.W., Steward, R. and Chen, X.M. (2005) Methylation as a crucial step in plant microRNA biogenesis. *Science*, **307**, 932–935.
48. Azuma-Mukai, A., Oguri, H., Mituyama, T., Qian, Z.R., Asai, K., Siomi, H. and Siomi, M.C. (2008) Characterization of endogenous human Argonautes and their miRNA partners in RNA silencing. *Proc. Natl Acad. Sci. USA*, **105**, 7964–7969.
49. Wu, H., Ye, C., Ramirez, D. and Manjunath, N. (2009) Alternative processing of primary microRNA transcripts by Drosha generates 5' end variation of mature microRNA. *PLoS ONE*, **4**, e7566.
50. Morin, R.D., O'Connor, M.D., Griffith, M., Kuchenbauer, F., Delaney, A., Prabhu, A.-L., Zhao, Y., McDonald, H., Zeng, T., Hirst, M. et al. (2008) Application of massively parallel sequencing to microRNA profiling and discovery in human embryonic stem cells. *Genome Res.*, **18**, 610–621.
51. Landgraf, P., Rusu, M., Sheridan, R., Sewer, A., Iovino, N., Aravin, A., Pfeffer, S., Rice, A., Kamphorst, A.O., Landthaler, M. et al. (2007) A mammalian microRNA expression atlas based on small RNA library sequencing. *Cell*, **129**, 1401–1414.