MicroRNA expression profiling using microarrays

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Microarray technology is a powerful high-throughput tool capable of monitoring the expression of thousands of small noncoding RNAs at once within tens of samples processed in parallel in a single experiment. To conduct a genome-wide analysis of miRNA expression of normal and disease samples, such as cancer, and to distinguish expression signatures associated with diagnosis, prognosis and therapeutic interventions, we have developed a unique miRNA microarray assay on a CodeLink platform. The miRNA array consists of 4,104 probes printed in duplicate. This array can simultaneously profile more than 1,500 mature miRNAs and their corresponding precursors from 474 human and 373 mouse miRNA genes. The full protocol details of the miRNA microarray assay developed by our group are described here, including miRNA oligo probe design, array fabrication and miRNA target preparation (by reverse transcription of total RNA), target-probe hybridization on array, signal detection and data analysis. The assay is simple, can be easily standardized and allows the reproducible profiling of up to 24 total RNA samples within 24 h.

INTRODUCTION

Over 5,000 members of a new class of small noncoding RNAs (ncRNAs), named microRNAs (miRNAs)[1,2], have been identified in the last few years in vertebrates, flies, worms and plants, and even in viruses[3]. The discovery of miRNAs has broadened our understanding of the mechanisms that regulate gene expression with the addition of an entirely novel level of gene expression control. In humans, the miRNoma (defined as the full spectrum of miRNAs) comprises more than 700 mature miRNAs derived from 533 precursor sequences (Sanger miRbase version 10) that have been either experimentally or *in silico* cloned and the total miRNA number is soon expected to surpass the one-thousand mark[4]. Standard northern blotting, which is time consuming and requires large amounts of total RNA (5–20 μg for each blot) and handling of radioactive material, can otherwise be proposed for validating miRNA signatures highlighted by the microarray approach. This same role can be ascribed to the more simple and robust quantitative RT-PCR (qRT-PCR), which also has the advantage of requiring very small amounts of starting RNA. Compared to other high-throughput techniques, like bead-based flow cytometry[5], our approach is not only capable of specifically detecting closely related miRNAs but, on the other hand, it also does not require multiple steps for sample preparation of labeling and allows the simultaneous detection of a much larger number of ncRNAs, including miRNAs and other species of ncRNAs at the same time. To further understand the involvement of miRNAs in molecular biology and cancer pathology, our group has developed and reported the first miRNA microarray expression profiling method, using a novel detection platform and an easier methodology for global miRNA expression profiling that overcome the limitations of common methods[6].

Methods to profile miRNAs

The most commonly used high-throughput technique for the genome-wide assessment of miRNA expression levels in a large number of samples is represented by oligonucleotide miRNA microarrays (for review, see refs. 6–8 (**Fig. 1**). Several technical variants of miRNA arrays have been independently developed in the last few years, and the main differences between them are the following: the oligo probe design, the probe immobilization chemistry, the sample labeling and the microarray chip signal-detection methods. In the protocol presented here, the miRNA oligo probes are 40 nt long, designed on the sense strand of the miRNA hairpin structure to cover both arms for the detection of mature and precursor miRNA (**Fig. 2**). Probes can alternatively be designed containing sequences from either the mature miRNA product or its precursor, to detect the specific expression of just one of these functionally different forms. The oligo probes, modified with a 5′ amine C6 linker (which covalently binds probes onto the chemical matrix of the slide surface by the amine group and increases the accessibility of probes for target hybridization), are printed onto polymer-coated CodeLink-activated slides. We do not employ UV crosslinking for probe immobilization as performed by others[9], as it can mesh and hinder the target hybridization accessibility of the probes and because the number of molecules corresponding to each oligo probe immobilized on the slide substrate by UV crosslinking may vary from batch to batch, resulting in changes in probe concentration due to evaporation of solution on reused library plates. More importantly, compared to other methodologies, our miRNA microarray protocol requires minimal sample manipulation and a reasonable amount of starting material. A quantity of 2.5 μg of total RNA is directly reverse-transcribed with biotin-labeled random octomer primer to obtain labeled target cDNA, ready to be hybridized on the microarray slides (**Fig. 3**). Small RNA enrichment, by fractionation or amplification by PCR as described[5], is not applied in our protocol, to minimize biases and errors introduced by the different efficiencies of adaptor ligation on enriched small RNAs and PCR templates, and furthermore from the nonlinear amplification of PCR amplicons as targets. After hybridization of the biotin-labeled target cDNA with the probes on the array slides, the biotinylated target/probe complexes are stained by affinity binding of Streptavidin-Alexa 647 conjugate to the two biotin molecules present for each target cDNA. This allows an eightfold linear signal amplification (there are two biotin molecules per cDNA target and four Alexa 647 fluorophores per streptavidin)

with a substantial and reliable increase in sensitivity (**Fig. 3**), compared with RNA direct end-labeling using Cy-dye modified nucleotide analogs by ligation[9] or PCR amplification[5]. Signal detection is finally obtained by laser excitation of the microarray, to allow the measurement of signal intensity.

miRNA expression analysis can be assessed with the use of different microarray platforms or by alternative methodologies (**Table 1**). For example, the bead-based flow cytometric technique[5], characterized by highly specific solution phase probe/target hybridization kinetics, is able to profile only limited subsets of miRNAs (<100 miRNAs per profile) due to the limitations of the color-decoding system on beads. The qRT-PCR specific either for precursor miRNA[10] or active miRNA[11,12] is a highly sensitive methodology, requiring small amounts of starting material for the rapid and quantitative analysis of a subset of miRNAs, excellent for microarray data validation. The miRAGE approach, the genome-wide miRNA analysis with SAGE (Serial Analysis of Gene Expression)[13], can be used for both the analysis of known miRNAs and discovery of unknown miRNAs and requires large amounts of computational and bioinformatics efforts for the analysis of the obtained tagged sequences. Each of these techniques has strengths and weakness (**Table 1**), and the confirmatory use of a second independent technique is now mandatory to identify false (positive/negative) data for all the different assays undertaken.

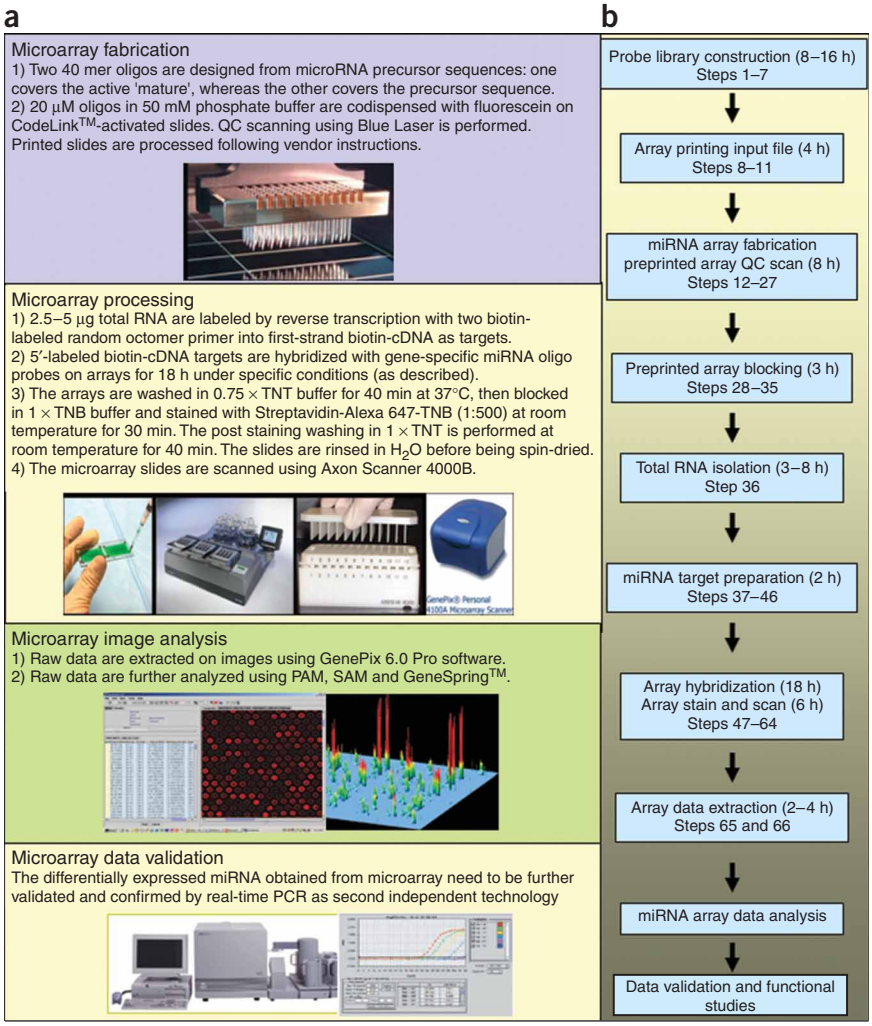

Figure 1 | Procedures and main steps of miRNA expression profiling. The methodology of microarray-based miRNA profiling described here and used in profiling studies on primary tumors is initially developed by Liu *et al.*[6] (**a**) This involves four main steps (presented in the left boxes): (i) microarray fabrication, (ii) microarray processing, (iii) microarray image analysis and (iv) microarray data validation by real-time PCR. (**b**) The protocol flowchart of the main steps for miRNA expression profiling.

Applications of miRNA profiling

We have successfully applied this method to study the global miRNA expression of normal human tissues, and we are able to identify specific microRNome expression signatures for each tissue analyzed[6]. The same genome-wide miRNA microarray platform has been used to analyze a large set of solid tumors and to compare them with their normal counterparts (for examples, see refs. 14,15). These studies revealed that cancer samples have variations in their miRNA expression pattern compared to normal samples, and that miRNA signatures allow tumors to be grouped on the basis of their tissue of origin. If routinely implemented in basic and clinical research laboratories, the genome-wide miRNA expression profiling can lead to the discovery of potential biomarkers and therapeutic targets.

After several years of miRNA profiling and the analyses of more than ten thousands primary tumors or cell lines on our miRNA arrays (for representative studies, see refs. 6,14–19), it has been proven that abnormal expression of miRNAs is a common feature of cancer cells. miRNA profiling achieved by various methods (**Table 1**) is therefore of high scientific relevance for the identification of miRNAs involved in the pathogenesis of human cancers and for the definition of human tumor signatures associated with diagnosis, staging, progression, prognosis and response to treatment of human tumors. Potentially, miRNA profiles can also be used in pharmacogenomics studies.

It is noteworthy that the protocol described here (**Fig. 1**) can be used not only for miRNA expression analysis on RNA isolated from fresh or frozen tissues, but also for miRNA profiling on RNA extracted from formaldehyde- or paraformaldehyde-fixed, paraffin-embedded (FFPE) tissues, bypassing the technical limitation of mRNA profiling from archived tissues, thus expanding the available sample sizes and overcoming difficulties in accessing patient's samples.

Experimental design

Probe design and preparation for miRNA microarray fabrication The current Ohio State University (OSU) miRNA microarray (version 4.0, microRNACHIPv4) contains a probe set of 4,104 oligos spotted in duplicate on CodeLink-activated slides (see below for further details on microarray fabrication). The detailed criteria

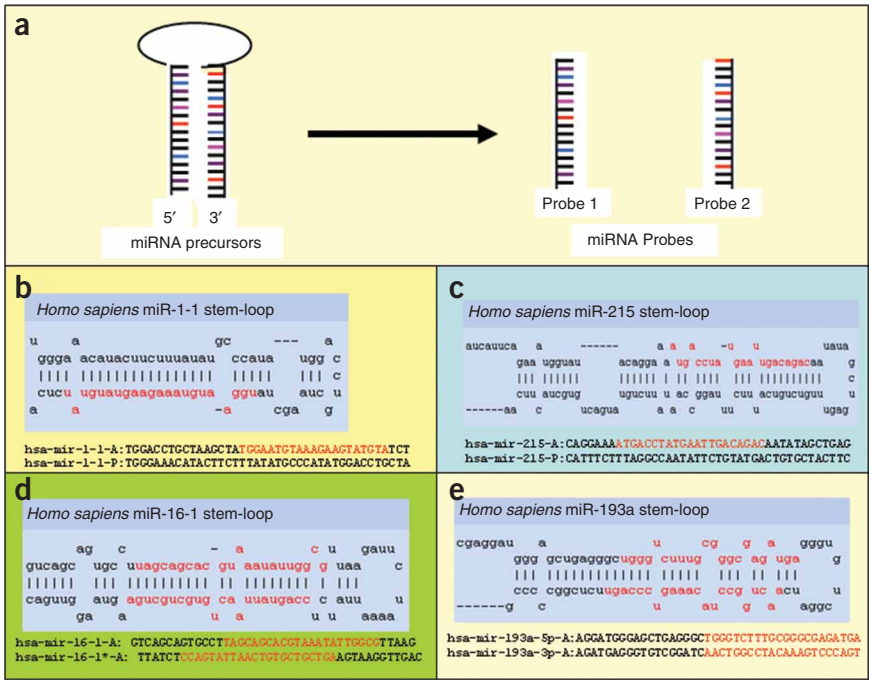

Figure 2 | miRNA oligo probe design and nomenclature of probe annotation. Two oligo probes are designed from both arms of the stem-loop of miRNA gene following the criteria described in text. (**a**) The colored horizontal short lines in stem-loop precursor (left) and probes (right) represent A, T, G and C nucleotides in DNA sequences. There are four categories of miRNA genes classified by position and nomenclature of active miRNAs in stem-loop precursors, as presented in panels **b**, **c**, **d** and **e**; one active miRNA encoded on either arm (**b** and **c**) or two different active miRNAs encoded on both arms of the stem-loop respectively (**d** and **e**). The sequences of stem-loop highlighted in red are the sequences of cloned and verified mature miRNAs present at Sanger miRbase. The two oligo probes designed, containing sequences from each miRNA stem-loop precursor and based on the criteria oligo probe design described as in Liu *et al.*[6] and in Experimental Design section), are listed in panels **b**, **c**, **d** and **e**. The mature miRNA sequence within oligo probe sequence is highlighted in red. The probe annotation and nomenclature of the two oligo probes from the same miRNA genes are named exactly as in Sanger miRbase with Affix at the end of probe name to differentiate two oligo probes designed from the same miRNA gene. The active miRNA oligo probes located at either 3′ (**b**) or 5′ (**c**) of stem-loop are named with affix-A, to differentiate from the other precursor's oligo probe named as Affix-P (panels **b** and **c** as examples). The oligo probes of two active miRNA encoded from the same miRNA gene are named as the same nomenclature of either (**d**) '*' or (**e**) '5p' and '3p' as in Sanger miRbase, with addition of Affix-A underneath stem-loop precursor in panels **d** and **e** as listed. As the miRNA nomenclatures in the miRbase are updated on a regular base, the probe nomenclatures and annotations in all output data files should be done extremely carefully.

of miRNA oligo probes designed on *Homo sapiens* and *Mus musculus* have been described[6]. The miRNA oligo probes were designed and derived from 847 miRNA precursor sequences (474 *H. sapiens*, 373 *M. musculus*) collected from the miRNA registry at http://www.sanger.ac.uk/Software/Rfam/mirna/ (version 9.0 January, 2007; and see ref. 16). In addition, other ncRNA probes were designed from the sequences of 481 human ultra-conserved sequences initially identified by Bejerano *et al.*[20], 700 human predicted miRNA sequences as identified[21–23] and 15 mouse and human tRNAs as found in the NCBI database at http://www.ncbi.nlm.nih.gov/entrez. Three *Arabidopsis thaliana* and 30 designed random oligo probes are used as negative controls.

miRNAs are small ncRNAs of 19–25 nt in size transcribed from 60 to 110 nt hairpin precursors. Two 40 mer oligo probes were designed from sense strand of both arms of hairpin structure of the

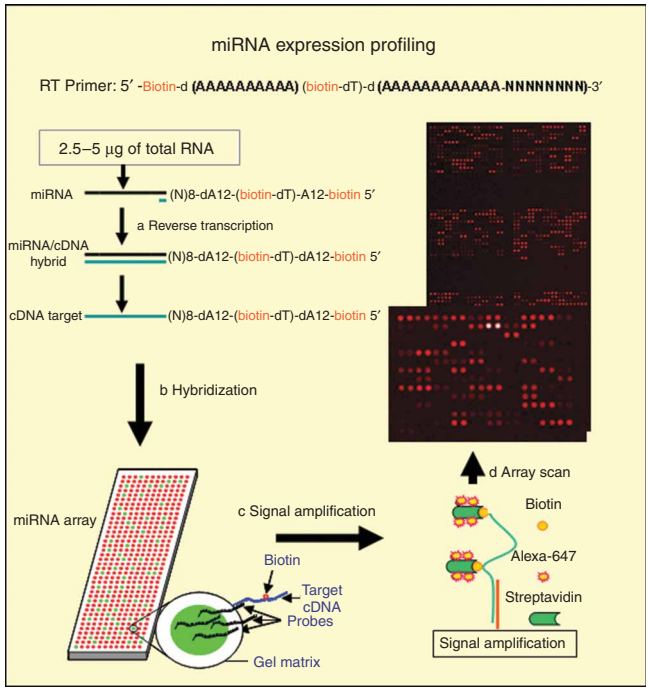

Figure 3 | Diagrams of detailed principle and procedure of miRNA expression profiling. The sequence of a random octomer RT primer labeled with two biotin molecules is shown at the top of this figure. As miRNAs are very short, the random octomer oligos (N8) designed and applied in this protocol have a complexity of 65,536 combinations (N^8, N = 4 of A T G and C), enough to cover and target the complexity of miRNA templates from total RNA in reverse transcription. Two biotin-modified nucleotides are included in the primer sequence, one at the most 5′ end and the second in the middle of the poly (dA) tail that extended from random octomer sequence and used for signal amplification by affinity binding of Streptavidin-Alexa 647 conjugates. The distances between the two biotins separated by poly (dA) and the internal biotin to the random octomer sequence (N8) represent the spacers used for affinity binding of two Streptavidin-Alexa 647 conjugates to the biotins for effective signal amplification. The four main steps of miRNA expression profiling as described (Steps 37–64). (**a**) Target labeling by reverse transcription of 2.5–5 µg of total RNA with biotin end-labeled oligo primer into single-strand biotin-labeled cDNA as miRNA targets (Steps 37–46); (**b**) hybridization of cDNA targets to oligo probes on the array slide (described in Step 52 and **Box 2**); (**c**) signal amplification by using Streptavidin-Alexa 647 conjugates to bind biotins on target hybridized on the array (Step 59). The signal is amplified eightfold for each hybridized target using two complexes of biotin–Streptavidin-Alexa 647 conjugates; and (**d**) array scan for signal detection performed by Axon array scanner (Step 63).

TABLE 1 | Examples of high-throughput methods for miRNA expression.

Type	Principle	Advantages	Disadvantages	References
miRNA microarray	miRNA chips printed with sense strand miRNA gene-specific oligonucleotide probes that hybridize with biotin-labeled cDNA targets, obtained by single-step reverse transcription of total RNA. Signal detection by staining with Streptavidin-Alexa 647 conjugates and detected by laser scanning	High-throughput genome-wide miRNA expression profiling is concomitantly achieved on large sample collections processed in parallel. The protocol is easily standardized	Solid-phase substrate affects probe-target hybridization kinetics and compromises specificity in detecting highly similar miRNAs. Microarray data are relatively compressed in fold changes compared to other methods	Ref. 6
Bead-based technology	Polystyrene beads coated with antisense oligo probes hybridize with biotin-labeled PCR amplicon dsDNA as targets. Staining with streptavidin–phycoerythrin, followed by bead flow cytometry for signal detection	Solution phase probe/target hybridization, which allows for higher specificity among closely related miRNAs	Low-throughput profiling with only small subsets of miRNA analyzed per experiment. Enrichment of small RNA by fractionation is necessary. More bias introduced in sample preparation by enrichment, adaptor ligation and PCR steps. Competitive hybridization between probes and targets of double-strand PCR amplicons occurs	Ref. 5
Stem-loop qRT-PCR for mature miRNA	Stem-loop primer cDNA reverse transcription followed by quantitative Taqman-based real-time PCR	High sensitivity (detection of 7 copies of miRNA per 25 pg of total RNA); high specificity; reliable quantitative results, useful in confirming microarray results; low cost	Low-throughput profiling for a genome-wide miRNA profiling assay	Ref. 11
qRT-PCR for precursor miRNA	Precursor gene-specific primers cDNA synthesis followed by quantitative real-time PCR	High sensitivity; high specificity; quantitative data	Low-throughput profiling for a genome-wide miRNA profiling assay	Ref. 10
miRAGE (SAGE)	Serial analysis of miRNAs gene expression by small RNA purification, tagging and cloning	Combination of cloning and expression profiling allows for the discovery of new miRNAs	Labor intensive. Enrichment of short RNA by fractionation is necessary	Ref. 13

same miRNA precursor sequence collected from Sanger miRBase database (http://microrna.sanger.ac.uk/cgi-bin/sequences/browse.pl). One represents the mature miRNA oligo probe in one arm and the other represents either the precursor oligo probe or a different mature miRNA on the opposite arm of the same miRNA gene (**Fig. 2**). The oligo probes are modified at the 5′ end with amine-C6 linker and purchased from either Sigma-Genosys or Integrated DNA Technology at 50 or 100 μM concentrations in H_2O. We designed two 40 mer oligo probes to cover both arms to profile the expression and relative ratio of both the mature miRNA and its precursor or the different mature miRNA discovered on the opposite arm of the same precursor (**Fig. 2a**). In general, the majority of miRNAs published in Sanger miRbase were cloned originally from one mature miRNA on one arm of the stem-loop precursor (**Fig. 2b,c**); however, in several instances, a second different mature miRNA was recently discovered and located on the opposite arm of the same stem-loop precursor (**Fig. 2d,e**).

Practically, the same criteria and strategies described above can be applied to design a custom miRNA array for studies on different animal research models (such as our dog version of the OSU miRCHIP array). Collection of species-specific miRNA precursors from Sanger miRbase represents the first step, followed by the identification and mapping of the full-length sequence of individual mature miRNA in the stem-loop precursor sequence (**Fig. 2**). The best 40 nt oligos including 19–25 nt of mature miRNA sequence, designed and selected on both arms of stem-loop precursor with minimal internal secondary structure and maximal similarity of Tm and G+C content to others, represent the oligo probes to be purchased.

Finally, for the miRNA array fabrication, the working oligo probes library is assembled and constructed in the concentration of 20 μM in 50 μM sodium phosphate buffer, pH 8.0, with 2 μM of sodium fluorescein, as detailed in Steps 1–7.

miRNA microarray layout and printing miRNA microarrays are fabricated on CodeLink-activated slides. The CodeLink-activated slides purchased from GE Healthcare (PN 300011) are hydrophilic polymer (containing *N*-hydroxysuccinimide ester reactive group)-coated microarray slides. The slides are coated with polymer only on one side considered as reactive side of 'CodeLink' to bind DNA. In preparation for miRNA array fabrication, each slide from the batch of 100 slides is labeled with unique ID in sequential numbers on the back of the slides (written by diamond pen), to identify every single array slide for the purposes of array quality control (QC) scanning, sample tracking of hybridization and in-house miRNA array inventory. We have labeled and named our miRNA version 1.0

arrays started from R0001, version 2.0 arrays started from T0001, version 3.0 arrays started from Y0001 and our current version 4.0 arrays started from P0001. All array design files of our previous generations of miRNA arrays mentioned above have been submitted and deposited in ArrayExpress database (http://www.ebi.ac.uk/arrayexpress). The ArrayExpress Accession Numbers are A-MEXP-86 for version 1.0, A-MEXP-258 for version 2.0 and A-MEXP-620 for version 3.0.

The design of the array layout and the printing protocol are based on the number of oligo probes in library and redundancy of individual oligo probes to be printed on the same array slide. Our current miRNA array (version 4.0) has 4,104 oligo probes in 11 Greiner 384-well plates. We print the array using 32 pins in 4 × 8 pin configurations, meaning that 32 subarrays (blocks) are printed on each single slide. Each oligo probe is printed in duplicate next to each other using the same pin on the same subarray. Each subarray is designed as 20 × 16 spots, meaning that on each row we can print 20 spots and that 16 rows are available to be printed for a total of 320 spots per subarray (**Fig. 4**).

Our '4 × 8 miRNA version 4.0' printing protocol is detailed in Steps 8–35, and the parameters and settings using GeneMachine OmniGrid software 'Gridder' version 4.2.1 on OmniGrid 100 arrayer are provided in **Box 1** and **Figure 5**. All technical details of operation on OmniGrid 100 arrayer and its software 'Gridder' for array designing and printing protocol can be found in the "User Manual" (http://www.genomicsolutions.com/).

Total RNA isolation The extraction of RNA from fresh or frozen tissues and cultured cells (see Step 36) should be performed using the Trizol reagent protocol (Invitrogen, 15596-018) or the newly developed mirVana kit (Ambion). In our experience from the studies of identification of differentially expressed miRNA and its target mRNA in cancer by expression profiling using the same total RNA, Trizol reagent extraction is popular and simple and maintains adequate quantities of both mRNA and small RNAs in the final extraction solution. The results of mRNA and miRNA arrays also correlate well with those of confirmation methods[16,18,19]. Total RNA isolation from FFPE tissues can be performed using newly developed RecoverALL Total Nucleic Acid Isolation kit (http://www.ambion.com/catalog/CatNum.php?AM1975).

miRNA target preparation The protocol of target preparation developed by our group (see Steps 37–46) is simple, easy and straightforward. We start with as little as 2.5 to 5.0 μg of total RNA. The labeled targets are directly generated as the first strand biotin-cDNA that is complementary to oligo probe on array, by using custom biotin-end-labeled random octomer DNA oligo primer (5′-biotin-$(dA)_{12}$-(dT-biotin)-$(dA)_{12}$-$(N)_8$-3′) in reverse transcription as described by Liu *et al.*[6] (**Fig. 3**). In our opinion, the protocols for miRNA target preparation developed by other groups may introduce more bias, due to the recovery efficiencies of small RNA fractionation in polyacrylamide gel and adaptor ligation, the non-linear PCR amplification and the bias of reporter molecule incorporation by 3′ extension labeling of polymerization[5,9].

miRNA array hybridization All miRNA array hybridization with miRNA-labeled targets is performed on the Tecan HS4800 hybridization station (see Steps 47–53). In general, the labeled targets of first-strand biotin-cDNA from 2.5 to 5 μg of total RNA are used to hybridize miRNA arrays in 6× SSPE/30% formamide at 25 °C for 18 h (**Fig. 6**). Hybridized slides are washed post-hybridization and signals on slides are detected using indirect detection of Streptavidin-Alexa 647 conjugate (Invitrogen/Molecular Probes) (see Steps 54–60 and **Fig. 3**). The processed slides are scanned in 10 μm resolution by using Axon 4000B Scanner (Molecular Devices) with 635 nm qred laser at Power 100% and PMT 800 setting (see Steps 63 and 64).

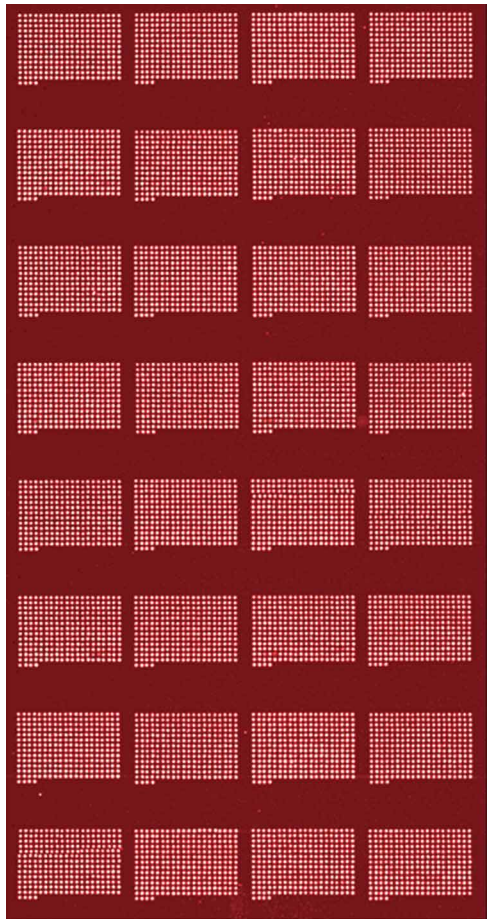

Figure 4 | The QC image of version 4.0 miRNA array. The white spots on the array slide are the oligo probes codispensed with fluorescein detected by 488-nm blue laser. A total of 4,104 oligo probes are printed in duplicate using 32 pins in 4 × 8 pin configuration (see **Fig. 5a**). The array consists of 32 subarrays (blocks). Each subarray, as designed in 20 × 16 spot configurations, contains 264 spots from 132 oligo probes that fill in the first $13^1/_2$ rows. The last $2^1/_2$ rows of each subarray are empty and can be used for further custom design.

The computational analysis of profiled miRNA raw data Microarray images are analyzed by using GenePix Pro, and postprocessing is performed essentially as described earlier[14]. Briefly, average values of the replicate spots of each miRNA are background-subtracted and subjected to further analysis. miRNAs are excluded from further analysis when less than 20% of their expression data values show at least a 1.5-fold change in either direction from the miRNA's median value. The threshold of 20% is used in a typical two-class experiment (i.e., reference sample versus test sample such as metastatic versus primary cancers). If the number of study classes in the experiment is higher, then the threshold can be lowered accordingly. miRNAs that are measured as absent (flag

BOX 1 | '4 × 8 MIRNA VERSION 4.0' PRINTING PARAMETERS

1. Plates and pins: the oligo probe library is assembled in 11 Greiner 384-well plates (see PROCEDURE), and a 4 × 8 pin configuration is used for printing (**Fig. 5a**).
2. Sample sequence: 11 Greiner 384-well plates are loaded into plate cassette in sequential order and the sample sequences on the plate in horizontal-fill for printing. The protocols for the sequences of plates and samples are created separately by 'Gridder' and integrated into printing protocol (**Fig. 5b**).
3. Origin offset: origin offset is the distance in the X and Y directions between slide origin calibrated and located where the printing pins will start on the slides. We set the origin offsets as 750 μm in vertical (Y) and 5,000 μm in horizontal (X), to better fit the printed array into the center of active areas of Tecan HS4800 hybridization chambers (**Fig. 5c**).
4. Array design: array design defines the size and shape of arrays. We design spot space as 200 microns in both horizontal and vertical, and 16 × 20 spots printed per subarray by the same pin. Each sample is printed in duplicates. Single array consists of 32 subarrays (or blocks) on each slide (**Fig. 5d**).
5. Dipping and cleaning: two dips at the same step sequentially are applied to each sample in well and each dip is for 1,000 ms and redip the same oligo applied after every 50 slides. The printing time on the slide is 0 ms as recommended by the user manual. Pin cleaning is critical to avoid any cross-contamination between the different oligo probes picked up by the same pin in the progression of slide printing. We clean the pins before each run, after each sample and after each plate using our default cleaning protocol. This consists of four repetitions of the following protocol in each loop: sonicate pins for 5,000 ms, wash pins for 2,000 ms and finally vacuum-dry pins for 1,000 ms. After the four loops of the cleaning protocol are completed, the pins are further vacuum-dried for 2,000 ms three times before moving to the next dips of a different oligo probe (**Fig. 5e**).
6. Blotting: blotting is performed by the pins being spotted onto a blotting pad, after the pins are freshly dipped into oligo probes from plates. Before printing onto the slides, we check and select 'blotting' in the protocol and blot 15 dots in 0 ms contact time on the blotting pad with the distance between dots as 200 μm (**Fig. 5f**).

<0) in a sample are thresholded to 1 (in log 2 scale) before normalization and statistical analysis. Normalization is performed using the quantiles method, implemented using the Bioconductor package/function Affymetrix/normalization[24,25], as it is currently the most widely accepted method for 1-channel microarrays. miRNA nomenclature is according to the miRNA database at Sanger Center. In a

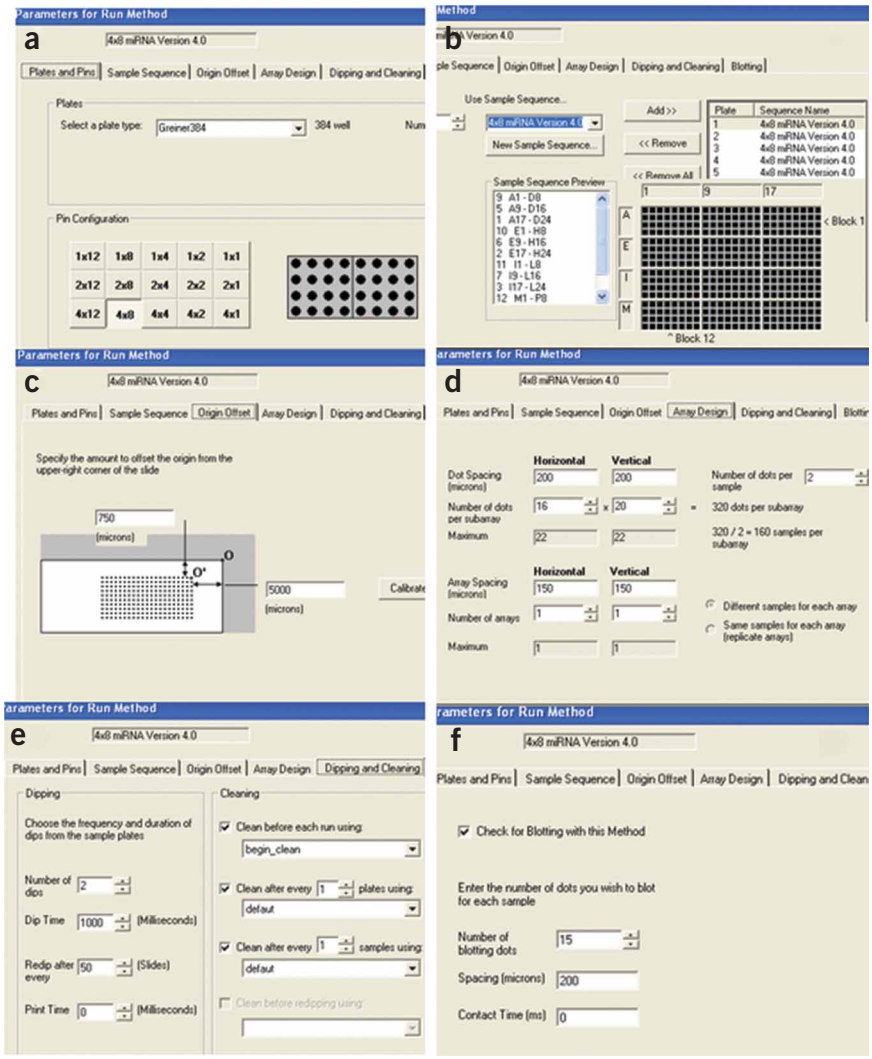

Figure 5 | '4 × 8 miRNA version 4.0' printing parameters. The printing protocol and parameters are described in **Box 1**. (**a**) The pin configuration for printing is 4 × 8 and 11 Greiner 384 plates. (**b**) The sequential order of the 11 Greiner 384 plates for printing, layout of printing blocks on Greiner 384 plate and the order of blocks to be printed in horizontal fill sequentially as blocks of 9 A1-D8; 5 A9-D16; 1 A17-D24; 10 E1-H18; 6 E9-H16; 2 E17-H24; 11 I1-L8; 7 I9-L16; 3 I17-L24; 12 M1-P8; 8 M9-P16 and 4 M17-P24. (**c**) The slide origin where the printing starts on slides is calibrated based on the active area of Tecan HS4800 hybridization chamber. The printing of this protocol starts at axis at horizontal 5,000 μm (X) and vertical 750 μm (Y) from slide origin. (**d**) The Subarray configuration of spot space is set to 200 μm horizontally and vertically in the layout of each subarray. (**e**) The frequency and duration of dips from sample plate during printing is shown on the left panel, and pin cleaning protocols applied during the array printing are described on the right. (**f**) This panel shows the blotting protocol of pins on the blot pad after each sample dip from plate, before the pins print on array slides.

two-class experiment (reference sample versus test sample), we identify genes that are differentially expressed using a random-variance *t*-test. The random-variance *t*-test is an improvement over the standard separate *t*-test, as it permits sharing information among genes about within-class variation without assuming that all genes have the same variance. The nearest neighbors and support vector machine classifier are used on miRNA expression profile to predict the class of future samples. The models incorporated the miRNAs that are differentially expressed as assessed by the random-variance *t*-test. The prediction error of each model is estimated by using leave-one-out cross-validation. The microarray datasets for publications are submitted and deposited in Array-Express (http://www.ebi.ac.uk/arrayexpress) using the online microarray data submission tool of MIAMExpress at http://www.ebi.ac.uk/miamexpress/.

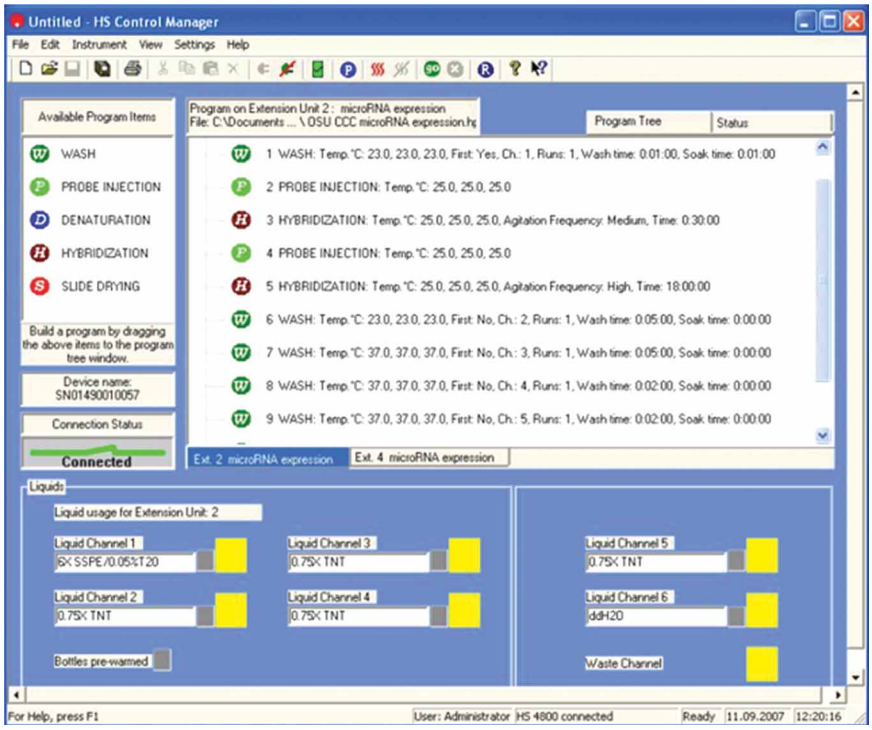

Figure 6 | Programmed miRNA array hybridization protocol. Automatic miRNA array hybridization is performed on Tecan HS4800 hybridization station. The ten-step standard procedure includes array precondition, prehybridization, hybridization and brief post-hybridization washing as described in the protocol of **Box 2**.

Validation of miRNAs results We perform the confirmation of microarray data mainly by quantitative real-time PCR analysis and rarely by northern blots. qRT-PCRs for miRNAs are performed as described for active molecule of miRNA or for precursor miRNAs[10], as our array can distinguish between the expressions of these two forms of miRNA. For northern blot analysis, 5–20 μg of total RNA is used for each sample to run on a 15% polyacrylamide denaturing (urea) Criterion precast gel (Bio-Rad), and then transferred onto Hybond-N+ membrane (Amersham Pharmacia Biotech). The blots are performed as described[26].

MATERIALS

REAGENTS

- 20× SSC (Sigma, S6639-1L)
- 20× SSPE (Sigma, S2015-1L)
- 5 M sodium chloride (Sigma, S5150-1L)
- 1 M Tris-HCl, pH 7.6 (Sigma, T-2788)
- Bicine (Sigma, B8660-1KG)
- Taurine (Sigma, T8691-100G) ! **CAUTION** May cause irritation to eyes, respiratory system and skin. Use gloves and safety glasses.
- Fluorescein (Sigma, F-6377)
- Anhydrous monobasic sodium phosphate (Sigma, S-3139)
- Dibasic sodium phosphate, heptahydrate (Sigma, S-9390)
- Formamide (Sigma, F-9037) ! **CAUTION** May cause harm to the unborn child. Causes severe eye irritation and is readily absorbed through the skin. Use gloves and safety glasses.
- 50× Denhardt's solution (Sigma, D-2532)
- 0.5 pmol μl−1 of (5′-biotin-$(dA)_{12}$-(dT-biotin)-$(dA)_{12}$-$(N)_8$-3′) custom random octomer oligonucleotide primer for reverse transcription (RT) reaction (Sigma Genosys)
- Tween-20 (Sigma, P-7949) ! **CAUTION** May cause eye and skin irritation. as well as respiratory and digestive tract irritation. Use gloves and safety glasses.
- Ethanol, absolute (Sigma, E7023-500ML) ! **CAUTION** Highly flammable and may cause irritation to respiratory tract, eye and skin. Keep container tightly closed. Keep away from sources of ignition.
- Isopropanol (Sigma, I9516-500ML) ! **CAUTION** Highly flammable and causes irritation to eye. Vapors may cause drowsiness and dizziness. Keep container tightly closed and keep away from sources of ignition. Avoid contact with skin and eyes and rinse immediately with plenty of water in case of contact. Use gloves and safety glasses.
- Chloroform (Sigma, C2432-500ML) ! **CAUTION** May cause irritation to eye and skin. May cause depression of the central nervous system, cardiac disturbances, reproductive and fetal effects, cancer based on animal studies and irritation to respiratory and digestive tracts. Use gloves and safety glasses and work under a fume hood.
- Xylene (Sigma, X2377-500ML) ! **CAUTION** Flammable liquid and highly poisonous. Vapor harmful if inhaled. May be fatal if swallowed. Avoid contact with eyes and clothing. Use with adequate ventilation. Keep container tightly closed. Wash thoroughly after handling. Use gloves and safety glasses and work under a fume hood.
- Superscript II RNase H^- reverse transcriptase (200 U μl^{-1}) (Invitrogen, 18064-014)
- 5× first-strand buffer (Invitrogen, 18064-014)
- 0.1 M dithiothreitol (DTT) (Invitrogen, 18064-014)
- 10 mM dNTP mix (Invitrogen, 18427013)
- Streptavidin-Alexa Fluor 647 conjugate (Invitrogen/Molecular Probes, S21374)
- 1× PBS, pH 7.4 (Invitrogen/LTI, 10010-023)
- Trizol reagent (Invitrogen, 15596-018; size 200 ml) ! **CAUTION** Corrosive to the eye and may cause severe damage including blindness. Causes skin burns. Components of the product may be absorbed into the body through the skin. Toxic if inhaled or swallowed.
- 10% SDS (Fisher, BP2436-1) ! **CAUTION** May cause severe irritation to eye, skin and respiratory and digestive tracts. Use gloves and safety glasses.

- NaOH (Fisher, S320-1) ! CAUTION Extremely corrosive and poisonous. Causes burns along the digestive and respiratory tracts. Use gloves and safety glasses and work under a fume hood.
- NEN blocking reagent (Perkin-Elmer, FP1020)
- Nuclease-free H_2O (Ambion, 9915G)

EQUIPMENT

- Sterile, nuclease-free conical tubes (15 and 50 ml) (Fisher, 14-959-49A)
- CodeLink-activated slides (GE Healthcare, 300011)
- Bioarray processors (GE Healthcare)
  - Bioarray rack (GE Healthcare, cat. no. 600010)
  - Small reagent reservoir (GE Healthcare, 600011)
  - Medium reagent reservoir (GE Healthcare, 600012)
  - Large reagent reservoir (GE Healthcare, 600013)
  - Bioarray removal tool (GE Healthcare, 600015)
  - Bioarray position tool (GE Healthcare, 600016)
- Pipette tips, sterile, RNase-free and aerosol-resistant (Ranin Instruments)
- Microcentrifuge tubes, sterile, RNase-free, 1.5 ml (Ambion, 12400)
- Gilson Pipetmans, 2,10, 20, 100, 200 and 1000 µl (Gilson)
- Greiner 384 high-base microtiter plate (E&K Scientific, EK-31101)
- Microtiter plate lid (E&K Scientific, EK-26101)
- PolarSeal foil adhesive tape (E&K Scientific, T592100)
- Powder-free gloves (Fisher, 19-130-2608C)
- NUNC 96-well plate (Fisher, 12-565-436)
- UltraJet All-Way spray duster (Fisher, 19-035-235)
- 0.8-µm Nalgene disposable filter unit (Fisher, 09-740-25AA)
- 0.22-µm Millipore sterile vacuum filter unit (Fisher, SCGP U11 RE)
- OmniGrid 100 Arrayer (Genomic Solution)
- TeMo liquid handler (TECAN US)
- HS4800 hybridization station (TECAN US)
- Axon Scanner 4200 (Molecular Device)
- Axon Scanner 4000B (Molecular Device)
- Computer (DELL) configured for Axon 4000B Scanner
- New Brunswick C24 incubator shaker (Fisher, 14-278-179) and utility carrier (Fisher, 14-278-128)
- Sigma/Qiagen centrifuge (4–15 °C) (Qiagen, 81010)
- Centrifuge plate rotor, 2 × 96 (Qiagen, 81031)
- ND-1000 Nanodrop (Nanodrop Technologies)
- Revco −80 °C freezer (Fisher, 13-989-169)
- Revco −20 °C freezer (Fisher, 13-990-206)
- Fisher isotemp refrigerator, 0–4 °C (Fisher, 13-986-120)
- Fisher model 205 and 210 isotemp water bath (settings 70, 65, 37 °C) (Fisher Scientific, cat. nos. 15-462-33 and 15-462-34, respectively)
- Mini centrifuge (Fisher, 05-090-128)
- Eppendorf centrifuge 5415D (Fisher, 05-40-100)
- Eppendorf centrifuge 5417R (Fisher, 05-406-8A)
- Eppendorf Vortex Genie 2 (Fisher, 12-812)
- Fisher dry bath incubator (Fisher, 11-718-4)
- Eppendorf vacufuge concentrator (Speedvac) (Fisher, 07-748-15)
- Pipette aid and disposable pipettes (Fisher)
- Pyrex glass container (Fisher, 08-741A)
- Desiccator (Fisher, 08-642-23C)

REAGENT SETUP

100 mM phosphate buffer, pH 8.0 (2×) Resuspend 0.69 g of anhydrous monobasic sodium phosphate and 25.46 g of dibasic sodium phosphate heptahydrate in 900 ml of distilled H_2O and adjust the pH of the solution to 8.0 by adding 100 µl of 10 N NaOH. Add more distilled H_2O to 1,000 ml volume and filter the solution using a 0.22-µm filter unit. The 100 mM (2×) phosphate buffer should be stored at 4 °C until use. ▲ CRITICAL Phosphate buffer concentration and pH improve spot morphology and oligo probe coupling on CodeLink-activated slides.

2 µM fluorescein solution Add 18.845 mg of fluorescein to 250 ml of H_2O to make a 200 µM solution. Then dilute 5 ml of this 200 µM solution in 495 ml of H_2O to make a 2 µM fluorescein solution that should be stored at 4 °C until use. ! CAUTION Fluorescein is light sensitive and should be protected from exposure to light.

Array-blocking solution (100 mM bicine and taurine) Dissolve 48.9 g of bicine and 37.5 g of taurine in 2,400 ml of H_2O and adjust the pH to 9.0 by adding ~40 ml of 10 N NaOH. Add more H_2O to the volume of 3,000 ml and store at room temperature (22–24 °C) up to 1 week or make fresh before use. ▲ CRITICAL Taurine is synthetic 2-aminoethanesulfonic acid used to block the *N*-hydroxysuccinimide ester reactive groups on the preprinted array slides to reduce background noise.

Array-washing solution (4× SSC/0.1% SDS) First dilute 20× SSC with H_2O to make a 4× SSC solution. Then add 30 ml of 10% SDS to 2,970 ml of 4× SSC and mix together. This solution should be made fresh each time.

1× TNT buffer (20 liters) 0.1 M Tris-HCl, pH 7.6/0.15 M NaCl/0.05% Tween-20. Add 2 liters of 1 M Tris-HCl, 600 ml of 5 M NaCl and 10 ml of Tween-20 into 17.39 liters of deionized H_2O. Mix well by stirring. Filter 1× TNT through a 0.2-µm filter. This solution can be stored up to 2 weeks at room temperature.

0.75× TNT buffer Add 250 ml of deionized water to 750 ml of 1× TNT buffer (from above) per 1,000 ml of buffer required. The 0.75× TNT can be stored up to 2 weeks at room temperature.

TNB buffer (0.5 liter) 0.1 M Tris-HCl, pH 7.6/0.15 M NaCl/0.5% NEN blocking reagent. Make 500 ml TN buffer by adding 50 ml of 1 M Tris-HCl, pH 7.6, and 15 ml of 5 M NaCl to 435 ml of nuclease-free H_2O. Then add 2.5 g of NEN blocking reagent to 500 ml of TN buffer and swirl the solution. Dissolve the blocking reagent in TN buffer in a 60 °C water bath to make TNB buffer. Filter TNB buffer through a 0.88-µm filter. Aliquot the TNB buffer into 50-ml conical tubes and store at −20 °C for up to 12 weeks. Thaw it immediately before use.

Streptavidin-Alexa 647 stock solution Add 1,000 µl of 0.1-µm filtered 1× PBS (pH 7.4) to the tube of anhydrous Streptavidin-Alexa 647 reagent. Vortex it briefly to dissolve dried powder in 1× PBS. This solution is light sensitive and should be stored at 4 °C in the dark and be used within 2 weeks.

1:500 Streptavidin-Alexa 647 working solution Centrifuge the Streptavidin-Alexa 647 stock for 1 min at 10,000*g* at room temperature to remove precipitates. Mix 100 µl of the Streptavidin-Alexa 647 stock solution and 50 ml of filtered TNB buffer for 12 slides to be processed. This working solution should be used within 15 min of preparation.

RT reaction cocktail mix The cocktail mix in 8 µl per RT reaction is composed of 4 µl of 5× first-strand buffer, 2 µl of 0.1 M dithiothreitol, 1 µl of 10 mM dNTP mix and 1 µl of Superscript II RNase H^- reverse transcriptase (200 U μl^{-1}) (Invitrogen). The reaction cocktail should be scaled up proportionally on the basis of the number of samples to be handled for reverse transcription. ▲ CRITICAL Make cocktail mixes fresh on ice and always add the reverse transcriptase as the last step. Extra reaction cocktail is needed in case of shortage caused by pipetting error.

PROCEDURE

miRNA oligo probe library construction ● TIMING 8–16 h

1| Transfer a 20 µl aliquot of 50 µM oligo probe from the 96-well mother plates obtained from the vendor into NUNC 96-well working plates (see Experimental design for further details of probe design), using a Tecan TeMo liquid handler with 96 tips.

2| Transfer 30 µl of 86 mM phosphate buffer, pH 8.0, containing 0.33 µM of fluorescein (25 µl of 100 mM phosphate buffer mixed with 5 µl of 2 µM fluorescein solution) into each well of the NUNC 96-well working plates using the TeMO liquid handler (to give a total volume of 50 µl per well, each containing 20 µM of oligo probes) and mix the oligo probes and phosphate salt buffer by aspiration.

3| Transfer a 10 µl aliquot of the 20 µM oligo probes from each well of the NUNC 96-well working plate into the wells of a Greiner 384-well high-base microtiter plate using the TeMo liquid handler (as described in Step 4). Repeat to produce five identical Greiner 384-well plates (sets).

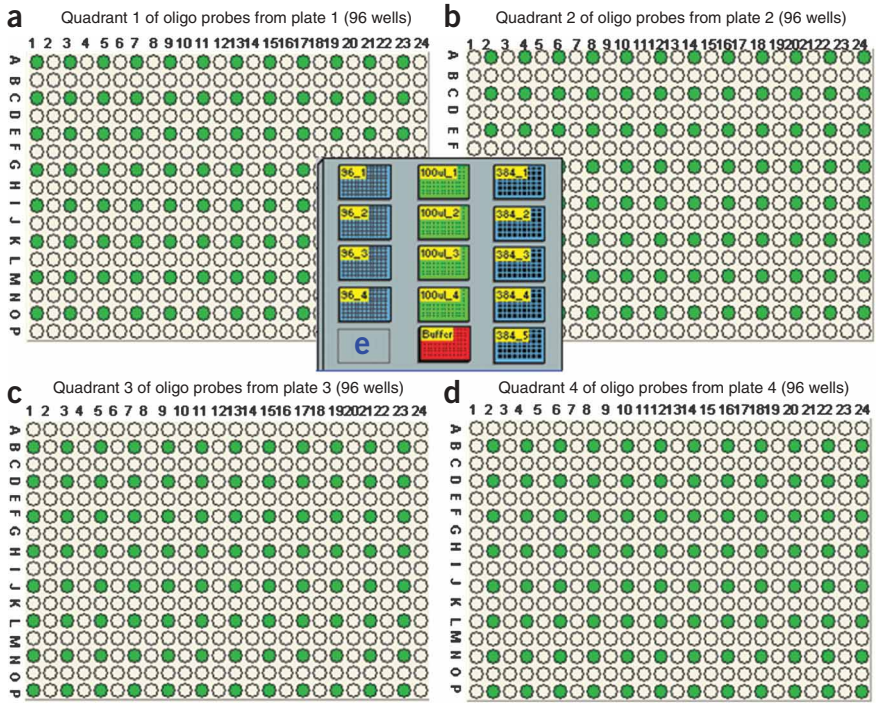

Figure 7 | Diagrams of plates reformatting in oligo probe library construction. The solid green circles in quadrants on Greiner 384 plate are the positions of oligo probes reformatted from NUNC 96-well plates. Every four NUNC 96-well plates with four different subsets of oligo probes in group are reformatted and assembled into one of Greiner 384-well plates. (**a**) The first quadrant of Greiner 384-well plate is for plate 1 of NUNC 96-well plate starting from A1. (**b**) The second quadrant of Greiner 384-well plate is for plate 2 of NUNC 96-well plate starting from A2. (**c**) The third quadrant of Greiner 384-well plate is for plate 3 of NUNC 96-well plate starting from B1. (**d**) The fourth quadrant of Greiner 384-well plate is for plate 4 of NUNC 96-well plate starting from B2. Oligo probes in 50 μM concentration from four different NUNC 96-well plates (left row) are dispensed into five identical Greiner 386-well oligo plates in 20 μM concentration diluted by 100 mM phosphate buffer, pH 8.0 (right row), performed during library construction. The plate assembling and reformatting from NUNC 96-well plates into Greiner 384-well plate are performed by TeMo liquid handler. (**e**) The layout of the working platform on TeMo liquid handler illustrated is that the left column has four NUNC 96-well plates in sequential order with different oligo subsets, the middle column has four boxes of 100 μl TeMo tips (green boxes) and one trough of 100 mM phosphate buffer (red box) as labeled, and the right column has five Greiner 384-well plates with identical oligo subset assembled from four NUNC 96-well plates.

4| Four quadrants on Greiner 384 plate are reformatted and assembled from four NUNC 96-well plates. The subset of oligos from four NUNC 96-well plates should be transferred to fill the wells of one Greiner 384-well plate, as described in detail in **Figure 7** as follows: Aliquots from the wells of NUNC 96-well plate 1 are transferred into the first quadrant of a Greiner 384-well plate, starting from position A1 on the Greiner plate. Aliquots from the wells of NUNC 96-well plate 2 are transferred into the second quadrant of the same Greiner 384-well plate, starting from position A2 on the Greiner plate. Aliquots from the wells of NUNC 96-well plate 3 are transferred into the third quadrant of the same Greiner 384-well plate, starting from position B1 on the Greiner plate. Aliquots from the wells of NUNC 96-well plate 4 are transferred into the fourth quadrant of the same Greiner 384-well plate, starting from position B2 on the Greiner plate.
▲ **CRITICAL STEP** Location and order of quadruplets of the oligo probes transferred from each NUNC 96-well plate to the same Greiner 384-well plates may be operator's preference. However, it is critical to record the order of plate assembly for library construction exactly, in order to create correct files for array printing and data extraction.

5| Apply the same procedure described in Steps 3 and 4 to assemble the miRNA printing oligo library plates from the rest of NUNC 96-well plates into the Greiner 384-well plates.

6| Cover and seal plates of working miRNA library with oligo probes by using PolarSeal foil adhesive tape.
▲ **CRITICAL STEP** Carefully cover and seal the library plates to avoid changes in salt and oligo concentration by liquid evaporation and any potential cross-contamination by handling the plates.

7| Store the plates of miRNA oligo probe library in order in a freezer set at −80 °C until use.
■ **PAUSE POINT** The constructed oligo library with buffer can be stored at the −80 °C freezer for months and used at any time for array fabrication.

miRNA array printing input file ● **TIMING 4 h**

8| Compile all individual Excel files provided by oligo vendor that correspond to NUNC 96-well plates in the order that the oligo library is constructed. Each Excel file contains probe ID, probe sequence and specific location of coordination with row and column of each probe.

9| Assemble and group Excel files of four NUNC 96-well plates into each of the Greiner 384-well plates (each containing oligos from four 96-well plates) in order. For example, Greiner 384-well plate 1 is grouped from NUNC 96-well plates 1, 2, 3 and 4 and so on.

10| Reformat and translate the probe coordinations from NUNC 96-well format into Greiner 384-well format as the oligo library constructed above. Here is the example also illustrated in **Figure 7**: NUNC 96-well plate1: the coordination of oligos at A1, A2 and A3… B1, B2 and B3…and C1, C2 and C3… will be reformatted as A1, A3 and A5… C1, C3 and C5 and E1, E3 and E5 of Greiner 384-well format plate and so on; NUNC 96-well plate 2: the coordination of oligos at A1, A2 and A3… B1, B2 and B3…and C1, C2 and C3… will be reformatted as A2, A4 and A6…C2, C4 and C6 and E2, E4 and E6 of Greiner 384-well format and so on; NUNC 96-well plate 3: the coordination of oligos at A1, A2 and A3…B1, B2 and B3…and C1, C2 and C3… will be reformatted as B1, B3 and B5…D1, D3 and D5 and F1, F3 and F5 of Greiner 384-well format and so on; NUNC 96-well plate 4: the coordination of oligos at A1, A2 and A3…… B1, B2 and B3…and C1, C2 and C3… will be reformatted as B2, B4 and B6…D2, D4 and D6…and F2, F4 and F6 of Greiner 384-well format and so on.

11| Assemble a consolidated input file in Greiner 384-well format from each individual Greiner 384-well input files in sequential order, based on the Greiner 384 plate orders of library construction, into one file and saved as both '*.xls file' and tab delimited '*.txt file'. The tab-delimited *.txt file with array printing protocol '4 × 8 miRNA version 4.0' applied together are used for creating the output sample tracking *.gal file by OmniGrid Arrayer control software 'Gridder'.
▲ **CRITICAL STEP** The 'Gridder' software only takes tab-delimited *.txt files for creation of the output sample-tracking GAL file (*.gal).

miRNA array fabrication and QC scanning ● **TIMING 8 h**

12| On the day the arrays are to be printed, take out the whole set of 11 Greiner 384-well oligo library plates from the −80 °C freezer and thaw them at room temperature.

13| Fill up the sonicator with deionized water up to a calibrated drawn line.

14| Load 100 labeled CodeLink blank slides onto OmniGrid 100 slide holders in a sequential order described in the user manual. The trademark of CodeLink should face up and on the left-hand side, opposite to the spring clip of the slide holder.
▲ **CRITICAL STEP** The labeled CodeLink-activated slides are loaded in order onto slide holders of platten as described in the user manual in order to track the quality of the preprinted array slides. The CodeLink-activated slides have to face up (the single side of polymer coated substrate) for array printing and oligo probe coupling onto slide surface.

15| Wipe and clean the blot pad gently with 0.01% SDS in water before and after each printing run.

16| Spin thawed oligo library plates at 1,000*g* for 2 min to remove all condensation from top sealed foil paper of plates.

17| Remove foil sealers from plate tops.

18| Load Greiner 384 plates in sequential order onto the plate cassettes of OmniGrid arrayer. To ensure accurate printing, it is critical that Greiner 384 plates are loaded in the cassettes such that sample well A1 is oriented toward the left rear corner of the OmniGrid arrayer when the plate is placed onto the platten. The plates should be centered between the sides of the cassette and pushed all the way to the back of the cassette. Once the plates have been loaded, the cassettes should be placed into the cassette stand, with the front or open side of the cassette facing the server arm.
▲ **CRITICAL STEP** To ensure accurate array fabrication, the Greiner 384 plates must be used in the sequential order as detailed in the oligo library printing file (from Step 11).

19| Close enclosure and turn OmniGrid control and server arm control boxes ON. The 40% humidity of enclosure is controlled and regulated by OmniGrid control box with a built-in humidity sensor, humidifier and dehumidifier.

20| Start 'Gridder' software, and after entering user name and password, the select Gridder operation menu will appear.

21| Test auxiliary devices, including wash and server arm devices of rinse station, dry station, sonicator, server arm grips, delid suction and plate nest grip, before running the real printing protocol.
▲ **CRITICAL STEP** Ensure that all auxiliary devices are working properly before the array fabrication start up.

22| Once all devices are ready and working properly, select printing protocol from 'Run Method' such as '4 × 8 miRNA version 4.0' and click OK. After verifying that the parameters in the programmed printing protocol are correct (see Experimental Design, **Box 1** and **Fig. 5** for details of information about the printing protocol and parameters), click the RUN button to view other partial-run parameters of the starting and ending sample plate numbers, starting and finishing at sample block numbers, starting and ending slide number from 1 to 100.

23| Also select 'miRNA version 4.0' plate sequence protocol created already and check the 'use the server arm to de-lid plates' function in the run parameters tab to complete the review.

24| By clicking the 'Start' button, the OmniGrid arrayer starts to run the protocol of '4 × 8 miRNA version 4.0' in a nonstop manner and completes full run of 100 fabricated miRNA arrays in 4 h.
▲ **CRITICAL STEP** Do not interfere with the machine during the array printing, stay with array printer as necessary to ensure the robot array works properly from the beginning to the end of the run.

25| Before unloading the entire batch of 100 preprinted slides from slide holders, a pilot QC scan of printed arrays, performed by 488-nm blue laser of Axon 4200 Scanner, detecting codispensed fluorescein from spots of printed oligo probes, is needed on the 50th and 100th printed slides within each batch (using the same technique as described in Step 27), to check both quality and spot morphology of printed arrays. Based on the scanned QC image data of those two slides, it is possible to refill the missed spot(s) precisely, without changing the position of the rest of the unloaded slides on slide holders, by customized printing if necessary.

26| Unload all 100 printed slides from slide holders into the slide boxes.

27| All 100 printed slides in the batch are QC-scanned in 20-μm resolution by 488-nm blue laser of Axon 4200 Scanner under scan power 100% and PMT650 (**Fig. 4**). All QC TIFF images scanned should be saved and used as reference to the following experimental profiling data on the same array. The preprinted arrays with 99.9% of probes printed are considered good arrays for profiling samples and passed for QC. The missed $<0.1\%$ oligo probes or bad spot(s) on QC image should be flagged correspondingly as 'absent' or 'bad' spot(s) on experimental profiling images and raw data exported into *GPR (GenePix Result) file and sent for further advanced data analysis.

miRNA array postprinting processes ● TIMING 3 h after overnight probe coupling
28| QC-scanned preprinted array slides are placed and positioned on the metal slide rack at every other slot position. The full racks with 15 preprinted slides are placed on top of the rack holder and the rack holder is placed into a saturated NaCl humidification chamber (set up by adding as much solid NaCl to water as needed to form a 1-cm deep slurry in the bottom of a plastic container fitted with an airtight lid), with 70% humidity at room temperature for oligo probe on CodeLink slide for coupling overnight.
▲ **CRITICAL STEP** The covalent coupling of oligo probes on preprinted slides should take a minimum of 4 h and a maximum of 48 h under 70% humidity chamber. Overnight incubation is optimum.

29| Prewarm 3 liters of array-blocking solution, 3 liters of array-washing solution and glassware containers at 50 °C at the end of the day for overnight incubation.
▲ **CRITICAL STEP** Prewarming blocking and washing solutions at 50 °C is critical. It enables the preprinted array slides to be blocked for reduced background on assayed array image.

30| Next day, transfer the preprinted array slides from the humidity chamber and immerse in a prefilled container with 50 °C prewarmed array-blocking solution. Incubate for 60 min at 50 °C in a chamber of New Brunswick C24 incubator shaker with no agitation to reduce background noise on assayed array.

31| Rinse array slides with deionized water three times in Pyrex glass containers.

32| Immerse the racks with array slides into prefilled containers with 50 °C prewarmed array washing solution. Wash at 50 °C for 30 min with agitation in the New Brunswick C24 incubator shaker chamber.

33| Rinse array slides with deionized water four times in separate Pyrex glass containers.

34| Spin-dry the rinsed slides by centrifugation at 1,000*g* for 1 min at room temperature.

35| The dried slides are ready for hybridization with labeled miRNA targets or array slides and can be stored in a desiccator at ambient temperature until use within 6 months of shelf life.
■ **PAUSE POINT** The preprinted array on CodeLink-activated slides can be stored for 6 months with no reduction of image quality.

Total RNA isolation ● TIMING 3–16 h
36| Total RNA isolation is performed based on the instruction of commercially available kits of either Trizol for fresh or frozen tissues and culture cells, or RecoverAll Total Nucleic Acid Isolation kit for FFPE tissues (see Experimental Design for further details of total RNA isolation). Quantify the RNA samples by applying 1.2 μl of eluate to Nanodrop ND-1000 for OD reading and store the RNA sample at −20 °C until use.

▲ CRITICAL STEP Ensure that the total RNA is not contaminated with Trizol reagent. Contamination of total RNA with Trizol can reduce the enzymatic activity in reverse transcription. Ensure that no column procedures are applied in total RNA isolation that can remove all small RNA under size threshold of 200 nt wt-off (miRNAs are 19–25 nt).
■ PAUSE POINT RNA sample(s) can be stored at −20 °C until use.
? TROUBLESHOOTING

miRNA target preparation ● TIMING **2 h**

37| Prepare each total RNA sample for target preparation in individual 1.5-ml RNase-free Eppendorf tube.

38| In the tube, mix 2.5–5 μg of total RNA in 10 μl of RNase-free H_2O with 2 μl of 0.5 μg μl^{-1} primer (**Fig. 3**) (5′-biotin-$(dA)_{12}$-(dT-biotin)-$(dA)_{12}$-$(N)_8$-3′) together in a total volume of 12 μl.
▲ CRITICAL STEP To ensure the data are comparable, the amount of total RNA starting material should be the same in ongoing parallel experiments whatever possible. In our standard operating procedure, 5 μg of total RNA is optimum for additional sensitivity in detecting miRNAs expressed at a low level. Mixing the reaction mixes by pipetting is essential.
? TROUBLESHOOTING

39| Incubate the total RNA with primer in a closed tube in 70 °C water bath for 10 min to allow the primers and miRNA templates to anneal and hybridize each other specifically, and then transfer the reaction tube immediately onto ice.
▲ CRITICAL STEP Reaction takes place at 70 °C for high efficiency and specificity, without secondary structure on RNA templates.

40| Briefly centrifuge reaction tube for 5 s at 10,000*g* at room temperature to collect sample and condensation to the bottom of the tube and immediately place the tube on ice.

41| Add 8 μl of preprepared RT reaction cocktail mix (See REAGENT SETUP) to 12 μl of total RNA/primer mix prepared in Steps 38 and 39.
▲ CRITICAL STEP To minimize variability of the RT reaction, it is extremely important to ensure that all reagents in RT reactions are from the same RT reaction cocktail mix.
? TROUBLESHOOTING

42| Mix the reaction mixture gently by pipetting up and down several times and briefly spin the tube for 5–10 s if air bubbles appear in the reaction mix during mixing.
▲ CRITICAL STEP Mixing reaction mixes by pipetting is critical. Air bubbles in the reaction mix can affect the efficiency of RT reaction.

43| Incubate tightly closed tube for 90 min in a 37 °C water bath so that RT reaction synthesizes biotin-labeled first-strand cDNA as targets for detection on miRNA microarray.

44| Centrifuge the tubes for 5 s at 10,000*g* at room temperature to collect the sample to the bottom of the tube, and put tube onto ice.

45| Add 3.5 μl of 0.5 M NaOH/50 mM EDTA into the 20 μl RT reaction mix and incubate at 65 °C for 15 min to denature the DNA/RNA hybrids and degrade single-strand RNA templates.
▲ CRITICAL STEP Ensure that the RNA templates have been degraded completely to avoid competitive hybridization to the targets with oligo probes on the array.

46| Add 5 μl of 1 M Tris-HCl, pH 7.6, to neutralize NaOH in reaction mix from Step 45 at room temperature. Each labeled target in a total volume of 28.5 μl can be stored at −20 °C with clear label until use within the same day or next day.
■ PAUSE POINT The labeled biotin-cDNA targets can be stored at −20 °C for months until use.

miRNA microarray hybridization, signal detection and array scanning ● TIMING **24 h**

47| Turn Tecan HS4800 hybridization station power ON and activate 'HS control manger' software. Connect computer controller and HS4800 by click 'Connection Tab' on 'HS control manger'.

48| Prime all channels of Tecan HS4800 hybridization station starting from channel 6 with H_2O; then prime channels 5, 4, 3 and 2 in order with 0.75× TNT wash buffer. Finally, prime channel 1 with 6× SSPE/0.5% (vol/vol) Tween 20.
▲ CRITICAL STEP Ensure that the tubing of all channels are filled with solution to avoid any air in the tubing that may affect HS4800 performance and array data quality.

49| Insert hybridization chambers into chamber frame based on the instruction of the user manual. The hybridization chambers cover the top of the slides to form a closed hybridization chamber for target hybridization on the slide surface.

BOX 2 | MIRNA ARRAY HYBRIDIZATION PROTOCOL

1. Prime the chip in hybridization chamber at 23 °C with 6× SSPE with 0.5% Tween 20 (Channel 1) for 1 min and soak for 1 min.
2. Inject 95 µl of prehybridization mix of 6× SSPE/2× Denhardts/30% formamide into hybridization chamber at 25 °C.
3. Prehybridize at 25 °C for 30 min with medium agitation.
4. Inject 75 µl of hybridization mix of each labeled biotin-cDNA target in 6× SSPE/30% formamide into hybridization chamber.
5. Hybridization of probes and targets on array takes place for 18 h at 25 °C with medium agitation.
6. Wash the array slides and chamber, post-hybridization, with 0.75× TNT buffer (liquid channel 2) at 23 °C for 5 min to remove hybridization mix from hybridization chamber.
7. Perform more stringent washes with 0.75× TNT buffer at 37 °C for a total of 9 min from liquid channels 3, 4, 5 at 5, 2 and 2 min, respectively, to remove unbound target on the slide surface.
8. Rinse with water from liquid channel 6 at 23 °C for 30 s to remove and rinse the salts of 0.75× TNA buffer on the array slide surface and prepare to unload the array slides from HS4800.
9. End program.

See also **Figure 6**.

50| Load preprinted miRNA array slides into slide adapter first and then place slide adapter with array slides (CodeLink-labeled end of the slides toward you and facing up) onto the module heating plates.
▲ CRITICAL STEP Ensure that the array slides are positioned correctly and recorded and that all slides are facing up as well.
? TROUBLESHOOTING

51| Close chamber frame with inserted chamber and array slides by lowering the arm and pressing downward until a 'click' is heard. A closed gap between slide and chamber is formed that enables the targets in the hybridization mix to hybridize to oligo probes on the slide surface and liquid to flow over the active area of slide.

52| Select preprogrammed miRNA hybridization protocol 'microRNA expression' program (**Fig. 6**) and run the programmed protocol for the array slide hybridization with biotin-labeled miRNA cDNA targets as detailed in **Box 2**.
? TROUBLESHOOTING

53| Unload array slides from opened module of HS4800 and transfer array slides as quickly as possible into the slots of the Bioarray rack that is placed in large reagent reservoir and soaked in 37 °C prewarmed 0.75× TNT buffer.
▲ CRITICAL STEP To release slide from the HS4800, open chamber frame by lifting the arm slowly until the clamp is unlocked. Let the chamber frame remain in the unlocked position for a few seconds so that the array slides have a chance to be released from the chamber O-ring and then open the frame completely.

54| Once 12 array slides have been transferred into the bioarray rack, position the array slides on the rack using the bioarray position tool tooth side down, to immerse all array slides in 37 °C prewarmed 0.75× TNT buffer.
▲ CRITICAL STEP Avoid slides getting exposed to air, as slides drying affects image quality due to increased background noise.

55| To wash slides post-hybridization, transfer the large reagent reservoir containing the array slides in racks onto the platform of a 37 °C conditioned New Brunswick C24 incubator shaker. The array slides in the large reagent reservoir are washed in 37 °C prewarmed 0.75× TNT buffer at 37 °C for 40 min with agitation at 50 r.p.m.
? TROUBLESHOOTING

56| To block the array slides in 1× TNB blocking buffer, transfer each bioarray rack of 12 array slides into a 12-cell small reagent reservoir that has been prefilled with 4 ml of TNB buffer per cell and block the array slide at room temperature on the benchtop for 30 min, with tapping the bioarray rack several times up and down.
? TROUBLESHOOTING

57| To allow hybridized array slide signal detection and amplification by Streptavidin-Alexa 647 conjugates, prepare fresh Streptavidin-Alexa 647 staining solution during the blocking of array slides in 1× TNB blocking buffer by adding 100 µl of Streptavidin-Alexa 647 stock solution into 50 ml of TNB buffer in 1:500 dilution and mix staining buffer well by aspiration up and down several times.
▲ CRITICAL STEP Ensure that the staining solution is made fresh and is sufficient to cover the array slide.

58| Dispense 4 ml of 1:500 Streptavidin-Alexa 647-TNB staining solution into each cell of another clean and dry small reagent reservoir.

59| Transfer blocked array slides on the bioarray rack from block solution into small reagent reservoir prefilled with Streptavidin-Alexa 647 conjugate staining solution and incubate array slides at room temperature for 30 min on the benchtop with tapping rack 2–3 times up and down.
▲ CRITICAL STEP Tap slide rack to remove attached small air bubbles on the slide surface.
? TROUBLESHOOTING

60| For post-staining washing, transfer the bioarray rack from the staining reservoir into a large reservoir prefilled with 1× TNT buffer and wash the array slides on New Brunswick C24 Shaker at 50 r.p.m. for total 40 min at room temperature in two fresh buffer changes.

61| Rinse array slides on the rack briefly by immersing rack and array slide in distilled water to remove all remaining wash buffer on the rack and array slides.
▲ CRITICAL STEP This step is critical to wash away all remaining salts and detergents on the slide surface, that cause background noise.
? TROUBLESHOOTING

62| Transfer array slides from bioarray rack onto metal slide rack using bioarray removal tool and spin-dry the array slides at 1,000*g* for 1 min at room temperature.

63| Scan processed array slides one by one with Axon 4000B scanner using red 635-nm laser at 10 μm resolution with Power 100 and PMT 800.
? TROUBLESHOOTING

64| Save scanned array TIFF file. The scanned array image TIFF file is saved as 'array ID-sample ID-PMT 800-Date', and the data are saved and deposited into user-defined folder in the designated server.
■ PAUSE POINT After array slides are scanned, the data extraction can be done at any time.

Sample tracking and data extraction analysis ● TIMING 2–4 h

65| The sample tracking output GAL file of 'miRNA array version 4.0' for GenePix software particularly is generated by using 'Gridder' software based on the array-printing protocol applied and the consolidated text input file of the oligo library printed (please read the detail from OmniGrid 100 User Manual).

66| The output result data from each scanned array slide image are extracted by using GenePix Pro 6.0 software and miRNA version 4.0 GAL file. The GPR of raw data from each array slide is saved together in the same user-defined folder as the TIFF array image file for further advanced microarray data analysis (see Experimental Design for further details).

● TIMING
Steps 1–7, miRNA oligo probe library construction: 8–16 h for 40 NUNC 96-well plates
Steps 8–11, miRNA array printing input file: 4 h
Steps 12–27, miRNA array fabrication and QC scanning: 8 h
Steps 28–35, miRNA array postprinting processes: 3 h after overnight probe coupling
Step 36, Total RNA isolation from fresh or frozen cells or tissues: 3 h; total RNA isolation from FFPE tissues: 8–16 h
Steps 37–46, miRNA target preparation: 2 h
Steps 47–64, miRNA array hybridization, signal detection and array scanning: 24 h
Steps 65 and 66: Sample tracking and array data extraction: 2–4 h

? TROUBLESHOOTING
Troubleshooting advice can be found in **Table 2**.

TABLE 2 | Troubleshooting table.

Problem	Possible reason	Solution
No signal	Array slides loaded upside down on HS4800 results in no target hybridization of probes (Step 50)	Repeat experiments and ensure that slides are loaded correctly in Step 50
	Assayed array slides are loaded upside up in Axon 4000B scanner (Step 63)	Load assayed slide correctly in Axon 4000B and rescan it
	Wrong laser is chosen for array scanning (Step 63)	Choose Laser 635 nm for detection of Alexa 647 signals (Step 63)
	The total RNA sample is prepared using a column protocol that loses all miRNAs during the RNA isolation (Step 36)	Repeat experiments and isolate total RNA using Trizol protocol

TABLE 2 | Troubleshooting table (continued).

Problem	Possible reason	Solution
	Primer is not added to total RNA in Step 38	Ensure that primer is added for reverse transcription
Low signal	Insufficient targets for probe hybridization on array in Step 52	Check total RNA concentration and repeat total RNA extraction for certain targets if necessary
	Total RNA is contaminated by Trizol that inhibits the reverse transcription reaction (Step 36)	Repeat total RNA extraction. Check new RNA by Nanodrop and compare the spectrum of RNA with 260/230 ratio for determination of potential Trizol contamination
	Check lot number and enzymatic activity of Superscript II (SSII) reverse transcriptase	Using new SSII reverse transcriptase for RT reaction
	Mixing of reagents in RT reaction is not sufficient in Steps 38 and 41	Mix by pipetting several times in each step
High background	Post-hybridization washing in Step 55 is not correct concerning the buffer (0.75× TNT) stringency, temperature (37 °C) and timing (40 min)	Make sure that the 0.75× TNT is prewarmed and conditioned for overnight incubation at 37 °C in Step 55
	Array slides are insufficiently blocked in Step 56	Check TNB buffer and prepare fresh if necessary
	Post-staining washes may have been carried out with no buffer changes or incorrect buffer (should be 1× TNT), room temperature or timing	Follow protocol with two buffer changes in 40 min
	Salt and detergent remained on assayed slides in Step 61	Rinse slides sufficiently with water in Step 61 to remove salt and detergent before spinning dry
	Slides are exposed to air for too long and dried in all intermediate steps that cause high background	Avoid long exposure of slides to the air
Partial signal areas	Air bubbles are injected into hybridization chamber in Step 52	Avoid air injection during inject samples
	Air bubbles attached to array slide surface during staining in Step 59	Tap array slides rack up and down every 10 min during the staining in Step 59

ANTICIPATED RESULTS

The miRNA microarray platform described here is a sensitive, reliable and reproducible platform for miRNA expression profiling. A quantity of 2.5 μg of total RNA per each sample is sufficient for profiling (**Fig. 3**), and the detection assay gives the sensitivity of 1–3 copies per cell, with a linear dynamic range of 2.5 orders of magnitude and 90–94% specificity and reproducibility (coefficient variability $< 10\%$). We have profiled more than 10,000 biological and clinical samples on this platform. The data profiled on this platform have been published in more than 50 papers in peer-reviewed scientific journals. More than 90% of array data of differentially expressed miRNAs can be confirmed and validated by real-time PCR. This platform is sensitive and easily differentiates samples by miRNA global profiling signatures[14–16,27]. Furthermore, this platform, containing probes for both cloned and predicted miRNAs as well as for ultraconserved regions, is an ideal tool for custom identification of new ncRNAs, as proven by the recent identification of ultraconserved genes and their regulation by miRNAs[28].

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