

Video Article

Performing Custom MicroRNA Microarray Experiments

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Abstract

microRNAs (miRNAs) are a large family of ~ 22 nucleotides (nt) long RNA molecules that are widely expressed in eukaryotes¹. Complex genomes encode at least hundreds of miRNAs, which primarily inhibit the expression of a vast number of target genes post-transcriptionally^{2,3}. miRNAs control a broad range of biological processes¹. In addition, altered miRNA expression has been associated with human diseases such as cancers, and miRNAs may serve as biomarkers for diseases and prognosis^{4,5}. It is important, therefore, to understand the expression and functions of miRNAs under many different conditions.

Three major approaches have been employed to profile miRNA expression: real-time PCR, microarray, and deep sequencing. The technique of miRNA microarray has the advantage of being high-throughput, generally less expensive, and most of the experimental and analysis steps can be carried out in a molecular biology laboratory at most universities, medical schools and associated hospitals. Here, we describe a method for performing custom miRNA microarray experiments. A miRNA probe set will be printed on glass slides to produce miRNA microarrays. RNA is isolated using a method or reagent that preserves small RNA species, and then labeled with a fluorescence dye. As a control, reference DNA oligonucleotides corresponding to a subset of miRNAs are also labeled with a different fluorescence dye. The reference DNA will serve to demonstrate the quality of the slide and hybridization and will also be used for data normalization. The RNA and DNA are mixed and hybridized to a microarray slide containing probes for most of the miRNAs in the database. After washing, the slide is scanned to obtain images, and intensities of the individual spots quantified. These raw signals will be further processed and analyzed as the expression data of the corresponding miRNAs. Microarray slides can be stripped and regenerated to reduce the cost of microarrays and to enhance the consistency of microarray experiments. The same principles and procedures are applicable to other types of custom microarray experiments.

Video Link

The video component of this article can be found at <https://www.jove.com/video/3250/>

Protocol

1. Printing of custom miRNA microarrays

1. Print the microarray slides using the microarray core facility services at a university or a company. The quality of microarray fabrication is one of the most critical factors for the success of a microarray experiment. Try a few services if possible. Ideally, one would print 50-100 slides at a time, and all the slides will look identical, with well separated, individual spots.
2. For microarray support, we use the GAPS II coated slides.
3. For miRNA probes, we use the NCode Multi-Species miRNA Microarray Probe Set V2.
4. Dissolve all the oligonucleotides in 3 x SSC at 10 μ M and quadruply print them on the slides. Fix the slides by ultraviolet irradiation according to instructions for the GAAPS II slides. Label the slide with a diamond pen to mark the area and side with the printed probes. Store at 22°C.

2. Sample preparation

1. Any method or reagent can be used to isolate RNA, as long as it preserves small RNAs. We generally use Trizol (Invitrogen) to extract total RNA, with satisfactory quantity and quality of RNA preparations, as measured by A_{260nm} and A_{280nm} readings.
2. For RNA labeling, mix ~ 25 μ g of total RNA with 0.5 μ g of 5'-pCU-DY547-3'⁶ in 1 x buffer (50 mM HEPES, pH 7.8, 20 mM MgCl₂, 10 μ g/ml BSA, 10% DMSO) containing ~ 20 units T4 RNA Ligase 1, supplemented with ~ 10 mM DTT and ~ 0.02-0.03 final volume of the 10 x T4 RNA Ligase 1 buffer for ATP.
3. Allow labeling to proceed on ice inside a refrigerator for 2-24 hours. Precipitate RNA with 0.3 M sodium acetate and 3 volumes of ethanol. For efficient ligation and precipitation, dissolve total RNA at over 2 mg/ml, and keep the total ligation volume at ~ 20 μ l.
 1. If less RNA is available, the amount of input RNA and 5'-pCU-DY547-3' can be scaled down.
 2. If the RNA is very dilute, add carrier such as yeast tRNA to aid in precipitation.

4. Combine and label in total 1 μ g of reference DNA oligonucleotides corresponding to a subset of mammalian miRNAs using the Ulysis Alexa Fluor 647 Nucleic Acid Labeling Kit⁶ as a control for the hybridization process. Purify the labeled DNA using a CentriSep column and save in dark at -20°C.

3. Microarray hybridization

1. To use the slides for the first time, pre-hybridize the slides in a filtered solution of 3 x SSC, 0.1% SDS, 0.2% BSA for 30-60 min at 37 °C. Submerge them in water a few times, then in isopropanol. Dry the slides using a centrifuge with a slide adapter at 100 g for 5 min at 22°C.

Note: We use Corning microarray hybridization chambers and Erie Scientific's mSeries of Lifterslips to perform microarray hybridization.

2. Rinse the lifterslips in water, then in ethanol, before drying in air. Place a slide inside a microarray hybridization chamber base, and a lifterslip on top of the slide. The lifterslip will cover the probe area, with its white strips contacting the slide.
3. In the dark, spin down the precipitated, labeled RNA. Wash it once with 70% ethanol, and air dry the pellet. The pellet should be reddish.
4. Prepare a hybridization solution containing 400 mM Na₂HPO₄ pH 7.0, 0.8% BSA, 5% SDS, 12% formamide⁶ with 1/15-1/100 μ l of the purified, labeled reference DNA (2.4) per RNA sample. The amount of added reference DNA depends on how many different oligonucleotides are labeled (2.4). If there are over 100, then less dilution is needed.
5. Dissolve the RNA pellet well with ~ 60 μ l of the hybridization solution.
6. Add 20 μ l of water to each of the humidifying wells in the Corning microarray hybridization chamber base.
7. Add the mixture of labeled RNA and reference DNA to the slide. Use a thin pipet tip, gently touch the edge of the lifterslip, and allow the solution to enter the space between the lifterslip and slide through suction.
8. Place the hybridization chamber cover over the base, then seal the chamber with metal clips.
9. Put the whole chamber inside the plastic bag that comes with the chamber cassette from Corning.
10. Place the chamber cassette(s) inside a container with wetted paper towel; cover the container with plastic wrap and place it inside a 37°C humid, cell culture incubator for ~ 24 hours. These precautions (3.6, 3.9, 3.10) ensure that the hybridization solution does not dry out during incubation.

4. Post-hybridization processing

1. Disassemble the chamber cassettes one by one. Submerge a slide and its lifterslip in 2 x SSC at 22°C. The lifterslip will naturally fall off the slide. Place it in 0.8 x SSC. Wash the slides in 0.8 x SSC twice, then three times in 0.4 x SSC, 1-2 min in total. Dry the slides using a centrifuge with a slide adapter. Wash the lifterslips with water and save for re-use later.
2. Scan the slides with any suitable scanner, e.g., the Perkin Elmer ScanArray 5000 or Molecular Devices Axon GenePix 4000B microarray scanner. Scan at wavelengths close to 547 nm and 647 nm to obtain the corresponding image files.
3. Use programs such as BlueFuse to quantify pixel intensities in the input, image files from 4.2). Inspect individual spots with the program to exclude abnormal spots on the microarrays from further consideration. These abnormal spots usually arise from poor slide printing or slide handling after hybridization.
4. Use programs such as GeneSpring and Excel for data analysis and presentation. General considerations for microarray data normalization apply, which is beyond the scope of this paper.
5. Once satisfactory signals are obtained after 4.3), strip the microarray slides for reuse⁷. Rinse the slides in water, then immerse in pre-warmed, 1 mM NaOH and 0.1 x SSC in a staining dish at 62°C for 10-20 minutes. Repeat the incubation once. Wash the slides a few times in water for up to 60 minutes with gentle shaking at 22°C. Dry the slides using a centrifuge, and store at 22°C.
6. To use the regenerated slides, it is not necessary to pre-hybridize them again. Simply wash them in water followed by isopropanol, and dry the slides right before hybridization.

5. Representative Results:

We have largely followed the described procedures to profile global miRNA expression in thousands of samples, i.e., RNAs isolated from zebrafish to human specimen under many different conditions. **Figure 1** shows a scanned image of a microarray to demonstrate very precise and strong hybridization signals on the slide. The Pearson correlation coefficients between technical replicates of microarray hybridization are ~ 0.99⁷, indicating excellent reproducibility.

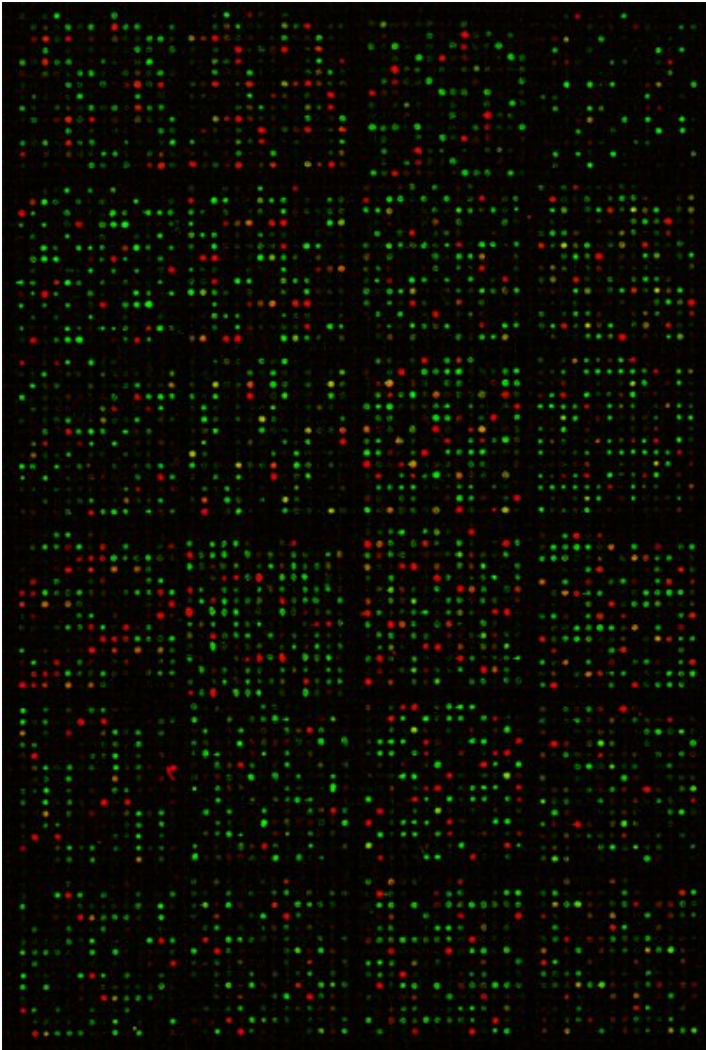


Figure 1 Composite image of a scanned miRNA microarray slide after hybridization. Red spots resulted from hybridizations by the reference DNA, green spots from the DY547-labelled RNA sample, while the yellow spots were from hybridizations by both the DNA and RNA to the same probes.

Discussion

Despite recent advances in deep sequencing technologies, microarray remains a viable choice for high-throughput analysis of DNA and RNA. Compared to deep sequencing, microarray experiments are cheaper, and a typical molecular biology laboratory can perform most of the experiments and data analysis in-house, which allows for flexibility and saves time. In the future, microarrays are likely well-suited to intensively interrogate sets of genes, e.g., all or a subset of the transcription factors in a genome or miRNAs, and the same principles and procedures presented here can be used in custom microarray experiments to study gene families besides miRNAs. Of course, a major drawback about microarrays is that sequence information must be available *a priori*. This is not a problem for most protein gene families in model organisms, although how many miRNA genes there are remain unclear. The probe set we have been using was designed based on miRBase⁸ Release 9.0 in October 2006. From recent studies^{9,10}, it is apparent that the most abundant and most biologically relevant mammalian miRNAs are already covered by this probe set.

There are no major technical challenges to perform custom microarray experiments. Based on our experience, the keys to the success of a microarray experiment are: 1) to obtain well-printed slides, 2) to prepare RNA of good quality and in sufficient quantity, and 3) to properly wash the slides after hybridization. Trizol reagent typically yields sufficient amounts of intact RNA for subsequent studies. Nonetheless, miRNA expression varies among different tissues and cell lines. Under most conditions, 20-25 µg of total RNA should give sufficiently strong microarray signals when labeled by the ligation method. Less RNA is required for miRNA-rich samples such as brain regions and neurons. If only 1-5 µg of total RNA or less is available, other labeling methods may be used, e.g., RNA labeling kits from Invitrogen, and they are compatible with the custom microarray platform described here. For the best comparisons, one should always label at least one replicate of all the control and experimental samples simultaneously and hybridize them to the same print batch of slides in a single experiment. This research design will minimize the contribution to differential microarray signals resulting from the variations in sample labeling and processing and in slide quality. The reference DNA (in red channel) serves as a control for the quality of slide, hybridization, and washing. A successful hybridization will give

clean and strong signals in red. If DNA signals are good but RNA signals are not, then one should trouble-shoot the steps of RNA isolation and labeling. For example, does the RNA have the ratio $A_{260\text{nm}}/A_{280\text{nm}}$ between 1.9 and 2.1? Is the pellet reddish in 3.3?

Most of the investment for microarrays may be for slide printing and scanning. A microarray printer and a scanner are often equipped by the core facility at many universities and medical schools. Printing microarrays in bulk and reusing slides and lifterslips will significantly reduce the cost. Reusing slides has the additional advantage of improving the consistency of microarray experiments⁷. We have reused slides more than six times and found the data quality still satisfactory. To ensure the best sensitivity for the most precious samples or samples with minimal input RNAs, however, it is still prudent to use fresh slides⁷.

Disclosures

No conflicts of interest declared.

Acknowledgements

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