Putting the ‘micro’ into microarrays: High throughput analysis of microRNA

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Abstract

MicroRNA (miRNA) plays an important role in the regulation of many cellular processes. Recent studies have indicated a regulatory role of miRNA in early development, cell proliferation, apoptosis, fat metabolism, and cell differentiation, while deregulation of miRNA has been linked to cancer development. A number of existing technologies, such as microarrays, are now being modified to allow for further investigation of miRNA. Due to the fact that miRNAs are small (21-22nt) the conversion of existing technologies has been non-trivial. In addition, miRNAs exist in various states in the cell (pri-miRNA, pre-miRNA, mature miRNA and recently mirtrons have been described). The UHN Microarray Centre has been evaluating a selection of emerging miRNA microarray platforms in terms of ease of use, robustness, reproducibility and validity of data. Each platform has specific requirements for miRNA isolation, whether it is miRNA isolation, total RNA isolation with miRNA enrichment, or total RNA isolation alone. For this reason, a number of isolation kits and protocols have been evaluated to determine the optimum protocol. Some miRNA microarrays consist of probes for mature miRNA alone while others include probes for pri-miRNA and pre-miRNA. A final consideration that must be made in this evaluation is how to handle to the data that can be obtained from the arrays. As with other microarray platforms, a large amount of data can be obtained from miRNA microarrays but consideration must be taken to determine the best steps in analyzing and interpreting the data. The UHN Microarray Centre has taken all of these factors into consideration in the evaluation of the various miRNA microarray platforms currently available to determine the optimum platform for our services. Here we present some of our findings from the evaluation of the Exiqon platform, one of two platforms which will be offered as as a service (the other being Agilent) by the UHN Microarray Centre.

Introduction

Despite evidence that miRNAs play a key role in the regulation of many cellular processes, there has been difficulty utilising high throughput analysis methods, such as microarrays for analysing this unique subspecies of RNA due to the small amount present, small size and different forms of miRNA that exist. Only 0.01% of the mass of total RNA is miRNA and of this there are 3 different forms; long primary miRNA (pri-miRNA), hairpin precursor miRNA (pre-miRNA) and short mature miRNA. An additional form of miRNA termed mirtrons has also been recently described (1-2). Typically, primary miRNA is first transcribed by RNA polymerase II which is then cleaved to form pre-miRNA (a stem loop structure 70-125nt in length). Drosha (an endonuclease) cleaves both strands of the stem loop structure creating 5’ phosphate and 3’ overhang of 2nt. The cleaved pre-miRNA is exported from the nucleus to the cytoplasm via RanGTP and exportin5 where the Dicer ribonuclease processes pre-miRNA into double stranded mature miRNA of 21-22 base pairs in length with a 3’ 2 nt overhang. The double stranded mature miRNA enters the RNA-induced silencing complex (RISC) where one strand of the mature miRNA causes RISC to cleave or repress a specific target messenger RNA (3). (Figure1)

There are a number of miRNA isolation kits and protocols that can be utilised to extract RNA from cells and tissues. The isolation kits vary in terms of whether they isolate total RNA with small RNAs remaining, total RNA that is enriched for miRNAs, or small RNAs only In order to perform a quality control analysis on the RNA that is isolated, a combination of chips run on the Agilent Bioanalyzer allow for both total RNA and RNA species to be analysed.

A number of different miRNA microarrays are available. Each of the different platforms differ in terms of probe design, type of miRNA profiled, species profiled, labelling protocols, and RNA requirements. The Exiqon miRCURY™LNA microRNA Array consists of capture probes of locked nucleic acids (LNAS) that are complementary to mature miRNAs from over 50 different organisms (Figure 2). Locked nucleic acids increase thermal stability and sensitivity which is extremely beneficial due to the small size of capture probes. The original labelling protocol provided as part of the Exiqon platform utilised 1-10 µg of total RNA which was labelled with fluorescent dyes in an enzymatic reaction. A new more sensitive labelling strategy has been recently released, but has not yet been validated by the UHNMAC. The standard Exiqon miRCURY LNA array platform has been evaluated in great detail in terms of ease of use, robustness, reproducibility, and validity of data.

Methods

RNA Extraction: Three different approaches were taken. 1) Total RNA (containing miRNA) was extracted from tissue culture cells using mirVana™ miRNA Extraction Kit (Ambion), microRNA Purification Kit (Norgen), miRNeasy Mini Kit (Qiagen), or TRIZOL® Reagent (Invitrogen). 2) Enriched small RNA was extracted from cells using the mirVana™ miRNA Extraction Kit (Ambion), or Purelink™ miRNA Purification Kit (Invitrogen). 3) miRNA was extracted from cells using the miRACLE™ miRNA Isolation kit (Stratagene). All extraction kits and protocols were followed as outlined by manufacturer’s instructions. RNA quality was determined by measuring concentration and absorbance ratios on Nanodrop and running samples on the Agilent Bioanalyzer.

Exiqon Array Analysis: 5 µg of TRIZOL extracted total RNA from different cell lines was labelled using miRCURY™ LNA microRNA Array Labelling kit following manufacturer’s instructions. The arrays were hybridised overnight following a user developed protocol utilising Agilent’s SureHyb hybridization chambers (http://www.exiqon.com/uploads/Agilent_SureHyb_protocol(2).pdf). Arrays were scanned using the Agilent Microarray scanner and data quantified using GenePix Pro 3.0 followed by analysis with GeneTraffic and SAM to determine statistically significant miRNAs. Further analysis was completed to determine reproducibility was completed using the Bioconductor package in the R programming environment. The same RNA was used for further testing using 1 µg of total RNA as starting material on Exiqon arrays with the same analysis completed.

Validation: miRNAs determined to have statistically significant expression changes using the 5 µg starting material was validated using TaqMan® MicroRNA Assay (Applied Biosystems) following manufacturer’s instructions.

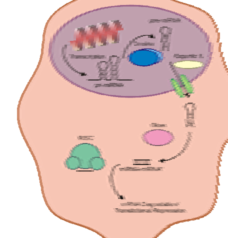

Figure 1: The canonical miRNA processing pathway.

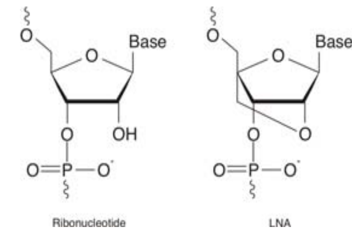

Figure 2: Locked Nucleic Acids (LNAs) are modified ribonucleotides.

Results

Table 1: Several different microRNA isolation strategies were tested and evaluated for performance.

Isolation Kit	# of Cells Required	Type of Isolation	Total RNA Concentration	Small RNA Concentration	RNA Isolated	RNA Integrity Number (RIN)	260:280	260:230
mirVana (Ambion)	5x10^6	Total RNA	1.3 µg/µl	N/A	177 µg	9.8	1.98	2.19
microRNA Purification Kit (Norgen)	6x10^6	Total RNA	1.5 µg/µl	N/A	75 µg	9.7	1.99	1.88
miRNeasy (Qiagen)	5x10^6	Total RNA	0.95 µg/µl	N/A	57 µg	9.7	2.02	2.24
TRIZOL (Invitrogen)	5x10^6	Total RNA	1.7 µg/µl	N/A	85 µg	9.5	1.93	2.25
mirVana (Ambion)	5x10^6	miRNA enrichment	N/A	0.47 µg/µl	47 µg	2.5	1.99	1.91
Purelink (Invitrogen)	6x10^6	miRNA enrichment	N/A	0.7 µg/µl	35 µg	8.1	2.02	1.57
miRACLE (Stratagene)	6x10^6	miRNA isolation	N/A	0.12 µg/µl	6.2 µg	N/A	1.8	1.43

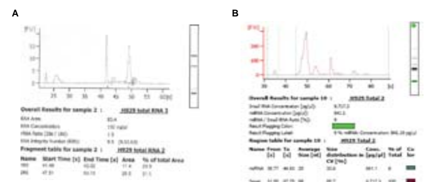

Figure 3: Use of the Agilent Bioanalyzer to assay TRIZOL® extracted total RNA. (**A**) shows the resulting electropherogram from the Nano kit looking at total RNA quality. (**B**) the same RNA sample run on the Small RNA Bioanalyzer kit indicating that 9% of the total RNA sample is comprised of miRNA

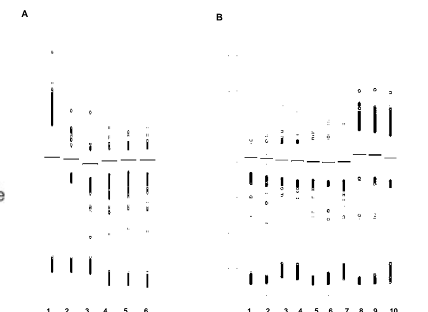

Figure 4: Boxplots created with Bioconductor (R programming language) showing reproducibility of Exiqon array data. (**A**) plots for data from arrays hybridized with 5 µg of total RNA. Arrays 1-3 are from the same lot and arrays 4-6 are from a second lot. Arrays were processed on different days with arrays 1 and 2 hybridised on the same day, 3 on another day and arrays 4-6 on a third day. (**B**) data for the tests using 1 µg of total RNA. Arrays 1-7 were hybridized with 1µg of total RNA and arrays 8-10 with 5 µg total RNA. Arrays 1,2 5,6, 8, and 9 were from one lot, 3,4, and 10 from a second lot, and finally array 7 from a third lot.

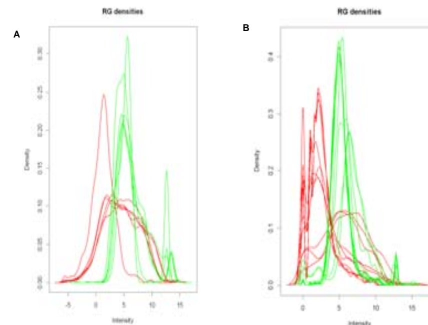

Figure 5: Signal intensities distributions for the red and green channels. (**A**) data compiled from 6 arrays where 5 µg of total RNA was labeled and hybridized to each channel. (**B**) comparison of 1 µg to 5 µg starting material. The green channel was hybridized with 1 µg of the same RNA for all arrays. For the red channel, 7 arrays were hybridised with the product 1 µg of total RNA and 3 were hybridised with 5 µg.

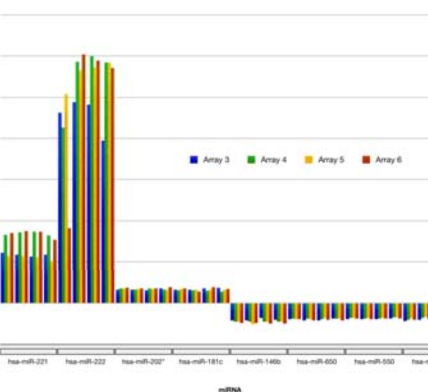

Figure 6: Fold changes of miRNA determined to be statistically significant between two cell lines from 4 arrays. Arrays 1 and 2 were not included in statistical analysis as there was a clear difference in these arrays in terms of reproducibility after analysis with R. Each miRNA is spotted onto each array 4 times and the fold change for each spot is shown.

Discussion

Microarray-based miRNA profiling is faced with several challenges. First and foremost, miRNAs are small nucleic acids that are actually shorter than the typical oligonucleotide probes used in many microarray platforms. Due to the small size of miRNAs, microarray probes have to be limited to small 21-22 bases in length (unless a unique probe design such as that in the Agilent platform is utilised). The short probe length diminishes the potential sensitivity and specificity of the assay. In order to overcome this limitation, the Exiqon miRCURY arrays utlise unique nucleotides called Locked Nucleic Acids which are suggested to improve binding affinity and specificity.

miRNA also presents a challenge to the investigator when it comes to isolation of these small nucleic acids. Typical column-based RNA extraction methods (RNeasy, Absolutely RNA etc…) tend to isolate total RNA that is depleted in miRNA and other small RNAs. Several extraction protocols were tested, each performing similar to expectations (Table 1). The ability to use common quality control metrics such as RIN scores from the Agilent Bioanalyzer is dependant on the type of RNA species isolated. The new small RNA kits from Agilent (Figure 3B) offer an additional way to assess the quality and quantity of miRNA and other small RNA species. While miRNA enrichment or purification strategies have shown promise, the efficiency of such methods and potential inconsistency can lead to problems with reproducibility of the array experiments. Exiqon recommends the simple extraction of total RNA including miRNA via TRIZOL. Our experience suggests that this is indeed a preferable method, however, potential contamination with phenols must be avoided.

Use of the Exiqon platform with either 1 or 5 µg of total RNA showed relatively consistent results even across array lots (Figure 4) however, the larger amount of starting material did show a non-surprising increase in overall signal intensities (Figure 5). With increased signals however, it is possible to detect slight variations that are less obvious with smaller amounts of starting material. Due to the increased signal and easier detection of expression changes, validation of microarray data focused at this point on the 5 µg array data. Using a modified t-test analysis with multiple testing correction (SAM - Significance Analysis for Microarrays, Stanford) several genes were identified as showing significant expression changes. The top four up-regulated and down-regulated miRNAs were identified and were examined for intra- and inter-array consistency (Figure 6). For each of these miRNAs, the consistency among for different arrays and among the 4 replicate spots on the array was found to be quite high. Validation of each of these genes was performed by TaqMan® microRNA Assay from ABI. Due to the low overall signal intensities, such validation is difficult, and was complexed by the fact that the cell lines chosen for this initial validation were both multiple myeloma cell lines with little expected difference. The two miRNAs with the greatest fold change difference (hsa-miR221 and hsa-miR222) validated well, whereas the other data was inconsistent and requires further analysis. The most significant down regulated gene (hsa-miR-146b) also validated. Such results suggest that reletivly stringent data filtering criteria may be required.

The UHNMAC is continuing its evaluation of the Exiqon miRNA microarrays. Exiqon has recently released a new version of their labelling kit involving two enzymatic reactions that will require between 250ng and 1 µg of total RNA to be used as starting material for subsequent labeling and hybridization. For some samples, even less total RNA (as little as 30 ng) may be used depending on the miRNA content of the total RNA. Testing is currently underway to determine the capabilities of this kit and similar analysis will be completed as a means of validating this protocol. Testing of the Agilent miRNA microarray is also in progress. The Agilent miRNA microarray consists of human mature miRNA species and as little as 100 ng of total RNA can be labeled in the two step enzymatic labeling process. The Agilent array is one a colour platform and is designed such that each slides consists of 8-15 000 feature arrays. The UHN Microarray Centre will be offering both the Agilent and Exiqon platforms for service projects.

References

1. Okamura *et al.* (2007) Cell. 130:89-100.
2. Ruby *et al.* (2007) Nature. 448:83-6.
3. Shingara, J. *et al.* (2006) RNA. 11:1461-1470.

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