Single color analysis of microRNA expression using the miRCURY LNA™ microRNA Array platform

Abstract

The sensitivity and specificity of the miRCURY LNA™ microRNA Array allow researchers to reach the full potential of the microarray technology in their microRNA profiling studies. By combining LNA™-enhanced Tm-normalized capture probes with market-leading microRNA coverage, a truly unique microarray platform has been created.

The high quality of the miRCURY LNA™ microRNA Array platform provides the flexibility to fulfill a variety of different experimental needs including challenges with difficult sample types, small RNA samples and specific experimental set-ups. Besides offering a dual color array protocol, the flexibility of the system has been further increased with the introduction of a robust single color protocol. Using the single color analysis approach, new samples can be added to previously performed experiments and analyzed in the same context enabling long-term/continuous studies to be performed for direct comparison without technical bias. The data presented here demonstrates that the array platform offers the high quality and reproducibility needed to accurately profile microRNAs in single color experiments. Analyses performed on different days with different lots of components produce highly similar results. In addition, microRNA data from the literature was reliably reproduced from a variety of tissue sources.

Taken together, the data presented here demonstrates that the miRCURY LNA™ microRNA Array platform offers the quality and flexibility for microRNA profiling in a wide variety of setups and that it truly is the perfect companion for your microRNA studies.

Introduction

MicroRNAs are small non-coding RNAs that play important roles as regulators of gene expression. They bind to the 3' UTR of their target mRNA thus inhibiting translation or facilitating mRNA cleavage (Bartel 2004; He et al. 2004). So far, more than 1,000 human microRNA sequences have been deposited in miRBase and new sequences are added with each new version of the data base (Griffiths-Jones et al., 2006).

MicroRNA expression patterns vary with tissue and cell type. In fact, some microRNAs are expressed almost exclusively in a particular kind of tissue. Altered microRNA expression profiles have been found to be associated with cell differentiation as well as to a number of different diseases including heart disease, neurological disorders and human cancers. This makes microRNAs good candidates for use as biomarkers.

Microarray analysis has quickly become a widespread method for microRNA expression profiling due to the high accuracy of the technology combined with a high throughput and low cost. Exiqon's miRCURY LNA™ microRNA Array platform is particularly well suited for microRNA expression analysis. It offers superior sensitivity, the highest coverage of known microRNAs and, as a unique feature, Tm-normalized capture probes, which ensures an equal detection of microRNAs regardless of the GC content of the sequence.

In the past, this platform has been mainly used for dual color microarray experiments. Here, we demonstrate the usefulness of performing single color experiments on the miRCURY LNA™ microRNA Array platform. We find that the platform offers excellent sensitivity, specificity and reproducibility. In addition, the results are in strong agreement with expression data available from the literature.

Results

Outstanding technical reproducibility

Low variation in replicates is an important factor for detection of low level differential expression. In addition, it reduces the number of technical replicates required for an adequate analysis. Hence, high reproducibility of a microarray platform is essential for long-term studies where different microRNA expression profiling experiments have to be directly and reliably compared.

In order to evaluate the technical reproducibility of the miRCURY LNA™ microRNA Array, we did four technical replicates of colon total RNA, labeled and hybridized on the same day. The median CV% between the capture probes of the four replicate arrays was 1.9 % and the Pearson correlation was >0.992 (Figure 1). This very low technical variability insures a high sensitivity to even the smallest regulations.

"median CV of 1.9% between arrays"

Figure 1

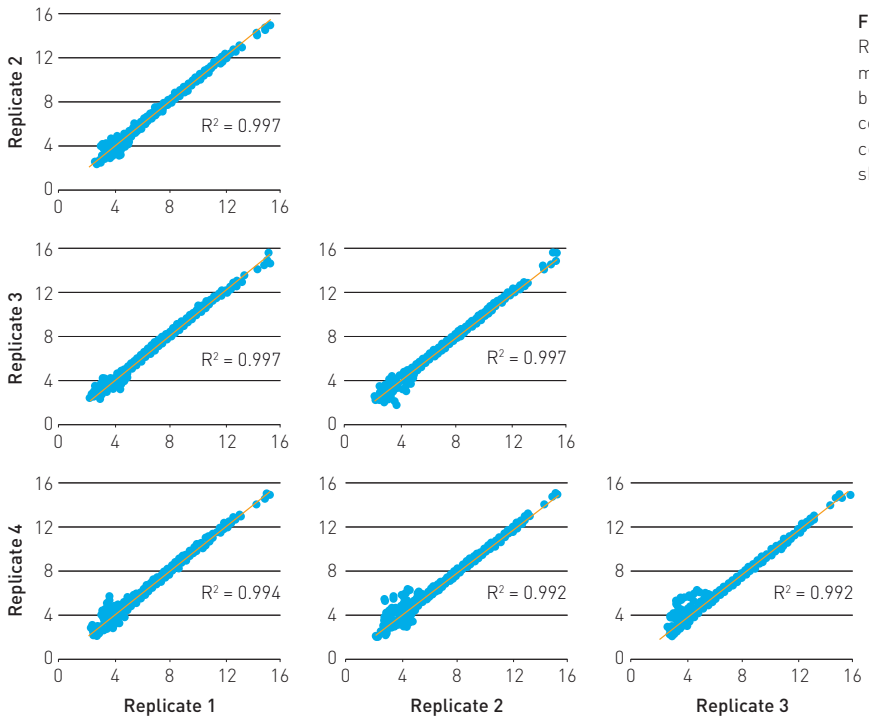

Figure 1. Four technical replicates of the colon total RNA performed on the same day on miRCURY LNA™ microRNA Arrays are compared. The correlations between each two of the replicates in all possible combinations are shown in the figure. The Pearson correlation coefficient for each combination is shown in the diagram.

Figure 2

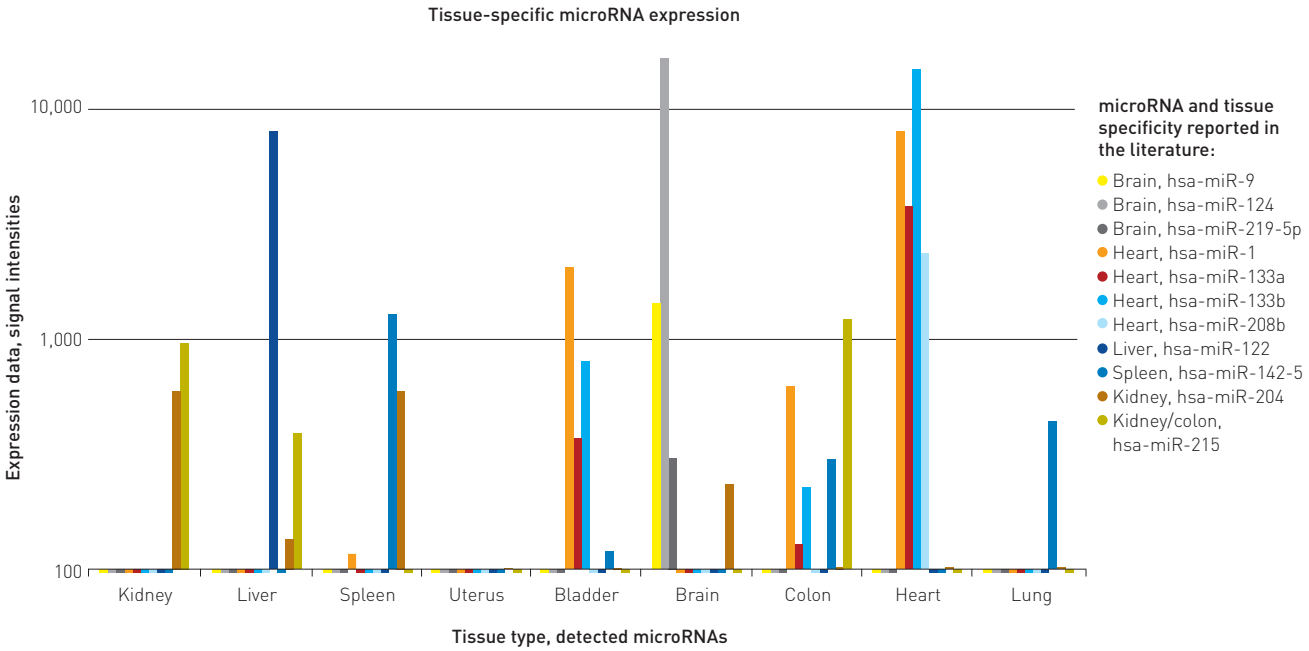

Figure 2. Expression of specific microRNAs known to be tissue-specifically expressed. The diagram shows which microRNAs were detected in particular tissues in the present study and the level of the microRNA detection in signal intensity. The bar coloring refers to in which tissue the microRNA has been reported found previously. Details on literature references are presented in the text below. As an example, hsa-miR-133a has been reported specific to heart muscle. The figure shows high presence of hsa-miR-133a in heart but also some minor expression in bladder and colon.

Reliable and sensitive detection of tissue-specific microRNAs

When comparing samples from different tissues, it is of great value to identify tissue-specific microRNAs as these have great potential as biomarkers. For example, they can be used to identify the source of cancers of unknown primary origin. The level of sensitivity determines the ability of the array to accurately detect all microRNAs expressed in a specific tissue of interest.

The level of sensitivity greatly depends on the technical variation, the sensitivity of capture probes and the ability of the array to detect microRNAs with different GC content. MicroRNAs with low GC content can be very challenging to detect due to the low Tm of the microRNA-capture probe complex. This problem has been overcome in the miRCURY LNA™ microRNA Arrays, which features LNA™-enhanced Tm-normalized capture probes that enable detection of all microRNAs including those of very low GC content.

In this study, we compared the results of microRNA expression analyses from various human tissues on the miRCURY LNA™ microRNA Arrays to results found in the scientific literature. We found a very good correlation between literature findings and signals on the array (Figure 2). We confirmed that hsa-miR-9, 124 and 219-5p are exclusively expressed in brain tissue as reported in several papers (Liu and Kohane, 2009; Cheng ĕt ăl., 2007). The four microRNAs typically found in heart: hsa-miR-1, 133a, 133b and 208b (Yang et al., 2007; Boštjancic et al., 2010) were all detected in high amounts, but also found in lower amounts in bladder and colon samples, most likely due to the presence of muscle tissue in these samples. The liver specific microRNA hsa-miR-122 (Chang et al., 2004) is exclusively detected in liver tissue, whereas hsa-miR-142-5p which has been found highly expressed in hematopoietic tissues (Chen et al., 2004) was found predominantly in the spleen, where the T-cells mature. In addition, it was found in lower amounts in lung as reported by Chen and co-workers. hsa-miR-204 has been identified to be highly expressed in kidney (Sun et al., 2004; Saal and Harvey, 2009) and this finding was reproduced using miRCURY LNA™ microRNA Arrays. However, we have also observed the presence of hsa-miR-204 in brain, liver, and spleen. hsa-miR-215 has primarily been reported from kidney (Kato et al., 2007) and colon (Song et al., 2010), but in our experiments we also detected hsa-miR-215 in liver. Landgraf et al. (2007) have reported increased levels of hsa-miR-215 in hepatic carcinomas.

In conclusion, the results obtained using the miRCURY LNA™ microRNA Array were very similar to those found in the literature.

Analysis of microRNAs expressed in clusters reveals a highly specific platform

A good way of evaluating the specificity of a microRNA array platform without using synthetic microRNA targets is to investigate if microRNAs are located in clusters. MicroRNAs in such clusters may be co-expressed and thus co-regulated.

In order to evaluate the precision of the miRCURY LNA™ microRNA Array platform, we have investigated the expression patterns of various microRNA clusters, from where the microRNAs are considered to co-express on the same pri-microRNA precursor (miRBase release 14). We found a very good correlation between the expressions of the different microRNAs found in the same clusters (Figure 3). We investigated three clusters where the microRNAs are located <1500 bases from each other. For all of these clusters, we see a close co-regulation indicating a high specificity of the array. It is worth noting that most of the microRNAs are also expressed from other chromosomal locations where they are arranged in different clusters of microRNAs.

However, the tight co-regulation of these microRNAs indicates that the majority of the transcribed/processed microRNAs arise from the three clusters under investigation (Figure 3).

Figure 3

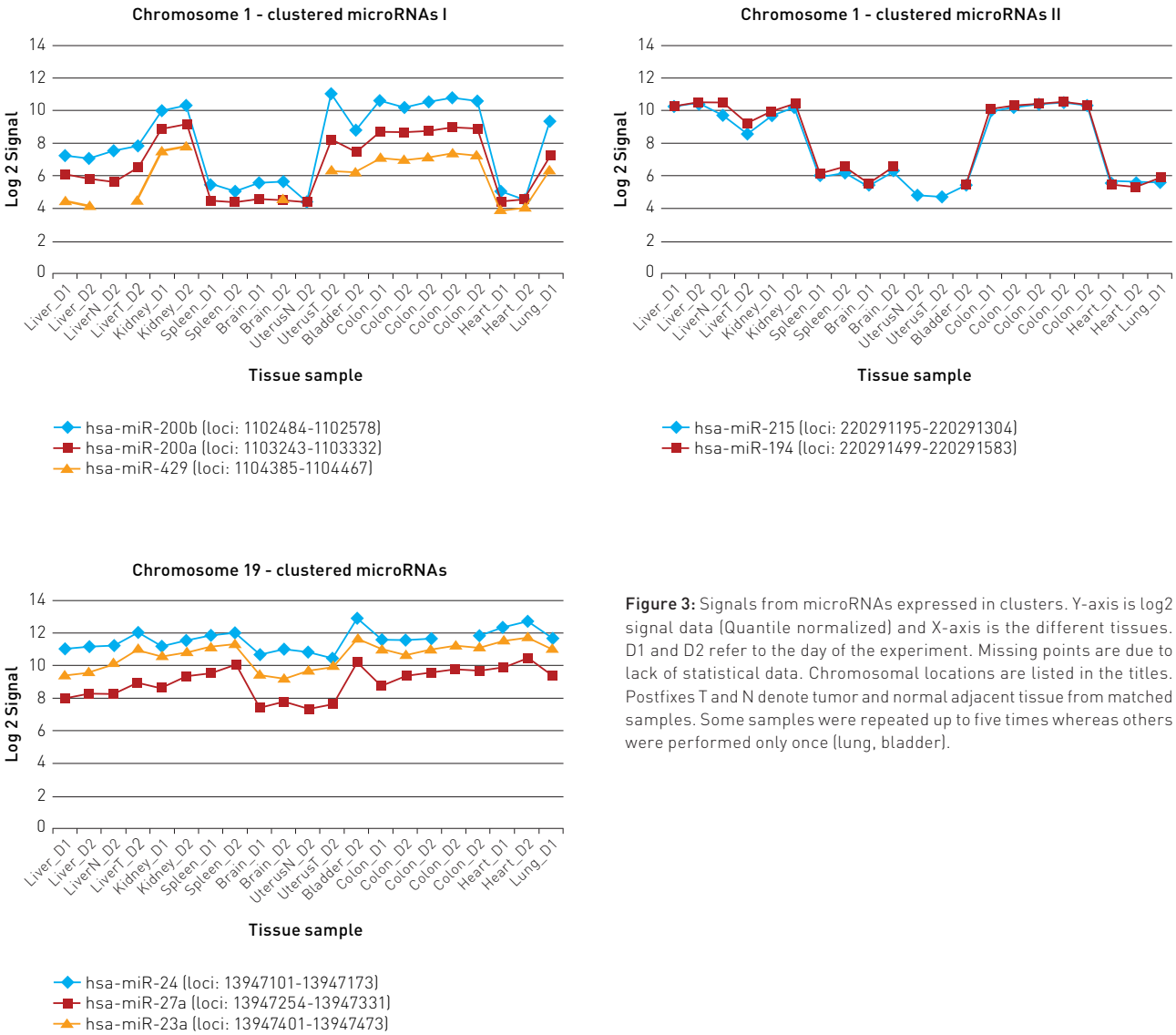

Figure 3: Signals from microRNAs expressed in clusters. Y-axis is log2 signal data (Quantile normalized) and X-axis is the different tissues. D1 and D2 refer to the day of the experiment. Missing points are due to lack of statistical data. Chromosomal locations are listed in the titles. Postfixes T and N denote tumor and normal adjacent tissue from matched samples. Some samples were repeated up to five times whereas others were performed only once (lung, bladder).

Figure 4

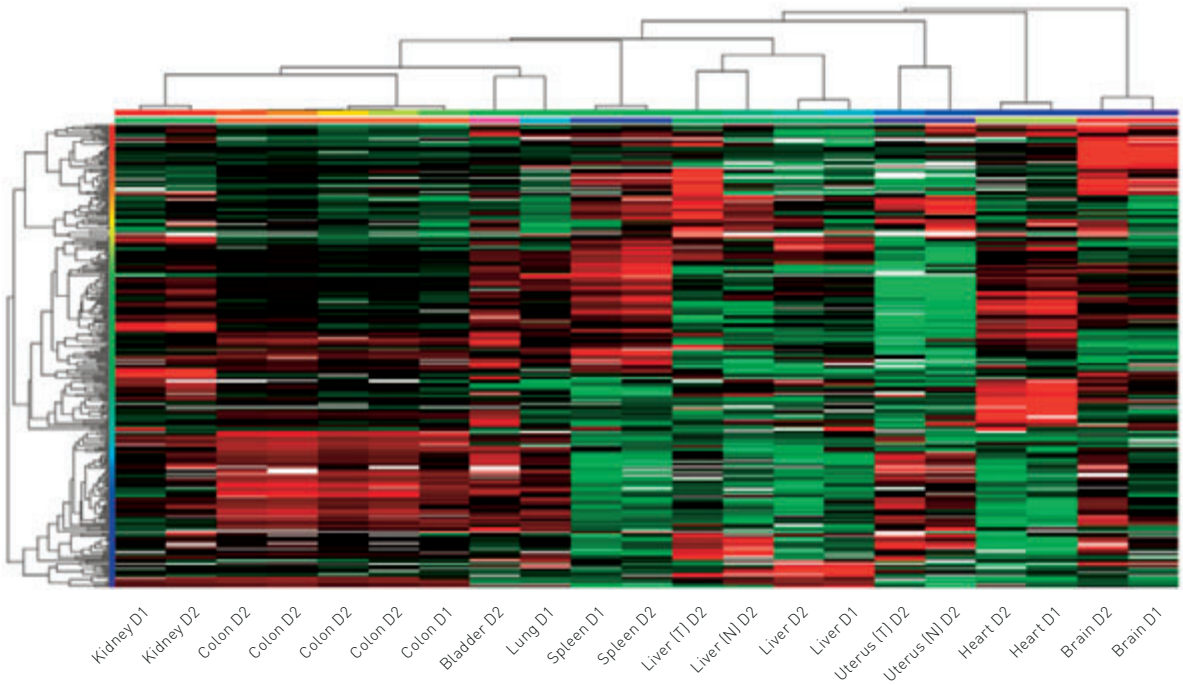

Figure 4. Heat map generated in miRCURY LNA™ microRNA Array customized Nexus Expression™ 2 software of microRNA expression data from all samples. The RNA samples and array capture probes are clustered using unsupervised hierarchical clustering. D1 and D2 refer to the day of the experiment. T and N denote tumor and normal adjacent tissue from matched samples.

Excellent day-to-day reproducibility

Unsupervised cluster analysis using the miRCURY LNA™ microRNA Array Nexus Expression™ 2 software was performed on the nine different tissues, using the log2 signal expression data (Figure 4).

As expected, we see that all replicate experiments (D1 and D2) performed on two different days cluster together, confirming that samples analyzed on different days, using different lots of components, give similar results.

Furthermore, all the different tissues separate nicely with brain, heart, and uterus forming distant clusters from all the intestines. A high call rate from the miRCURY LNA™ microRNA Arrays ensures a comprehensive data set for the generation of clustering information and the high degree of reproducibility ensures very low variation within groups.

Identification of differentially expressed cancer microRNAs

Next, we compared the results of normal and tumor tissue. From the matched samples, we see that even though the tumor and normal adjacent tissue cluster together, the two matched liver samples are different from the other normal liver sample tested, indicating that significant biological differences exist between individual patients (Figure 4). We have listed the top five up- and down-regulated microRNAs in the two matched tumor vs. normal tissue samples (Table 1). The uterus tumor is diagnosed as an endometrial adenocarcinoma and regulation correlated well with literature. We found that hsa-miR-200a/b and 205 were up-regulated compared to normal tissue. This is in concordance with recently published data by Ratner et al. (2010). hsa-miR-145 is the most down-regulated microRNA in uterus cancer resulting in a -1.5 fold regulation in liver.

Previously, hsa-miR-145 has been reported to be down-regulated in several different types of cancer. It has been suggested to be used as a cancer biomarker due to its postulated wide role as tumor suppressor (Sachdeva and Mo, 2010). The hsa-miR-29 family including hsa-miR-29a, 29b, and 29c was down-regulated in liver tumor, which is in agreement with previously published data (Su et al., 2009) (Xiong et al, 2010). The liver tumor was a hepatoblastoma from a three-month-old patient. This is a very uncommon malignant liver neoplasm occurring almost exclusively in infants and young children.

A previous study testing microRNA expression in this type of tumor only found very compressed differential expression between tumor and normal tissue (Magrelli et al., 2009). The most up-regulated microRNA, hsa-miR-10b (+1.99 log2 fold), was however also found to be 2.81 fold up-regulated on the miRCURY LNA™ microRNA Array.

In general, we have seen a very good correlation between data reported in the literature and data generated using the miRCURY LNA™ microRNA Arrays. These data indicate that the miRCURY LNA™ microRNA Array is able to reproduce and support data described in literature. Taken together, these results indicate that the miRCURY LNA™ microRNA Array can be reliably used in single color mode for the identification of up or down regulated microRNAs in tumors. The ability of the platform to identify such microRNAs makes it ideal for use in biomarker discovery.

Table 1

	Liver		Uterus	
Up regulated in tumor	hsa-miR-376c	4.614	hsa-miR-200b	6.636
	hsa-miR-381	4.477	hsa-miR-200a	3.820
	hsa-miR-205	4.445	hsa-miR-141	3.555
	hsa-miR-138	3.924	hsa-miR-205	3.228
	hsa-miR-377	3.787	hsa-miR-21	3.135
Down regulated in tumor	hsa-miR-29a	-1.941	hsa-miR-140-3p	-3.355
	hsa-miR-378	-1.990	hsa-miR-143	-3.765
	hsa-miR-29c	-2.022	hsa-miR-133b	-3.803
	hsa-miR-29b	-2.142	hsa-miR-125b	-3.948
	hsa-miR-139-5p	-2.969	hsa-miR-145	-5.530

Table 1. List of top five up- and down-regulated microRNAs in tumor vs. normal tissue. Numbers are displayed as delta between log2 of signals.

Conclusion

In conclusion, the miRCURY LNA™ microRNA Array used for single color analysis provides the researcher a unique and extremely powerful platform for accurate and reliable microRNA expression profiling. With unmatched reproducibility and sensitivity, the platform enables detection of subtle changes in microRNA expression, which is of critical importance for the study of disease development and in biomarker discovery.

We have compared RNA samples from a number of human tissues, using the miRCURY LNA™ microRNA Array, run in single color and analyzed using the Quantile normalization method with the miRCURY LNA™ microRNA Array Analysis Software package. We have demonstrated the usability of the single color approach by validating the results through comparison to the literature. In addition these results clearly proved the array capabel of producing highly accurate microRNA profile from complex samples such as FFPE. We further demonstrated the **exceptionally high technical reproducibility** of the method with CV-values below 2% and confirmed the ability to identify aberrantly expressed microRNAs. A number of microRNAs have been reported to be up-regulated in heart and brain compared to the intestines and we were able to confirm these findings, as well as those reported from the different intestines.

The specificity of the miRCURYLNA™ microRNA Array was evaluated by investigating microRNA expression clusters, in order to verify that the array was able to detect co-regulation of microRNAs found in the same cluster. Even though several microRNAs are expressed from different loci, we were able to demonstrate a very close co-regulation of microRNAs found within the same cluster

In summary, the high quality of the miRCURY LNA™ microRNA Arrays makes it highly compatible with single color microarray profiling using the Quantile normalization method. Adding this to a robust dual color protocol, this finding further extends the flexibility of the miRCURY LNA™ microRNA Arrays and its usefulness for microRNA profiling. The data analysis procedure is simplified by the intuitive miRCURY LNA™ microRNA Array Analysis Software package.

Materials and methods

RNA

All RNA samples used in this study were Ambion First Choice human total RNA which also contains the microRNA fraction. In addition, Ambion microRNA-certified total RNA from a number of matched tumor and normal tissue samples were included in the analysis. A list of the RNA used in the study is found in Table 2. Labeling and hybridization was performed in duplicate and the replicates were labeled and hybridized on two different days, using two different lots of labeling kits.

Table 2

Tissue	Batch number	Replicates
Liver	40000124	2
Liver, tumor + normal tissue	1034602	1+1
Kidney	908002	2
Spleen	095PO20303011A	2
Brain	105PO55201A	2
Uterus, tumor + normal tissue	600124	1+1
Bladder	10050088	1
Colon	055PO11102051	5
Heart	7040022	2
Lung	94002	1

Table 2. List of different types of RNA samples used in the experiments throughout the report. Batch numbers refer to Ambion First Choice stocks and in case of the liver and uterus tumor and normal samples, Ambion microRNA-certified total RNA.

RNA labeling

The RNA was labeled using the miRCURY LNA™ microRNA Power Labeling Kit (Exiqon, Denmark) according to the manufacturer's instructions. 0.5 µg total RNA from each sample was labeled with Hy3™.

Microarray analysis

The Hy3™-labeled samples were hybridized to the miRCURY LNA™ microRNA Array, 5th gen. – human, mouse and rat (Exiqon, Denmark). This array covers all annotated human microRNAs present in miRBase v. 14.0 as well as more than 350 miRPlus™ candidate microRNAs. The hybridization was carried out according to the manufacturer's instructions on a Tecan HS4800™ hybridization station (Tecan, Austria). Following hybridization, the microarray slides were scanned using an Agilent G2565BA Microarray Scanner System (Agilent Technologies, Inc., USA) and stored in an ozone-free (< 2.0 ppb) environment to prevent bleaching of the fluorescent dye. Image analysis was carried out using the ImaGene® 9.0 software of the miRCURY LNA™ microRNA Array Analysis Software package. Quantified signals were background corrected, using local background and normalized using Quantile normalization algorithm. Expression profiles of microRNAs across different human organs were clustered using the average Hierarchical clustering algorithm in the Nexus Expression™ 2 software of the miRCURY LNA™ microRNA Array Analysis Software package.

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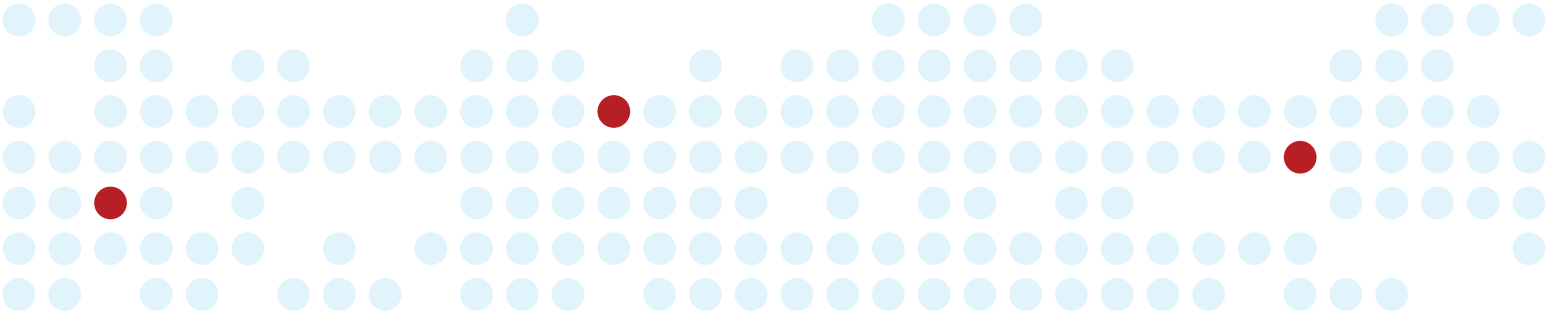

Contact information

Outside North America
Exiqon A/S · Skelstedet 16
DK-2950 Vedbaek · Denmark
Phone +45 45 660 888
Fax +45 45 661 888

North America
Exiqon Inc. · 14 F Gill Street
Woburn, MA 01801 · United States
Phone (781) 376 4150
Fax (781) 376 4152

www.exiqon.com

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