Urine microRNA profiling to discover biomarkers for nephrotoxicity

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Abstract

microRNAs represent the best described class of small RNAs (21-23nt) and have been shown to function as post-transcriptional regulators of gene expression. The high relative stability of microRNA in common clinical samples such as serum/plasma, urine and other biofluids, and the ability of microRNA expression profiles to accurately classify discrete tissue types and specific disease states have positioned microRNA quantification as a promising new biomarker for a wide range of diagnostic applications.

We have developed a genome-wide LNA™-based microRNA qPCR platform with unparalleled sensitivity and robustness. A single cDNA synthesis reaction is sufficient for full microRNA analysis without the need for pre-amplification. The platform is thus highly suited to high-throughput microRNA profiling from challenging clinical source material. Thousands of biofluid samples, including blood derived serum/plasma and urine, have been profiled in-house allowing the development of focused microRNA assay sets for the discovery of microRNA biomarkers for disease, toxicology and injury studies.

Here we demonstrate the applicability of urine microRNA profiling for the discovery of potential biomakers for nephrotoxin induced kidney injury using the Toxicology Focus microRNA PCR Panel. We also present our approach to the challenging aspect of data processing and normalization specifically for microRNA profiling in urine.

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Introduction

microRNA as disease and toxicology biomarkers

microRNAs constitute a class of small RNAs that function as post-transcriptional regulators of gene expression [1] that play important regulatory roles in most cellular and developmental processes and have been implicated in a large number of human diseases [2,3]. Since the discovery of extracellular and circulating microRNAs a few years ago, the study of microRNAs in biofluids such as serum, plasma, urine and cerebrospinal fluid has rapidly expanded [4]. Due to their wide-ranging biological potential and the fact that microRNAs seem to be relatively stable in readily available biofluids, these small 21–23 nt molecules are prime candidates for use as non-invasive biomarkers in molecular diagnostics of disease and other clinical conditions such as organ damage as well as pre-clinical toxicology and drug safety assessments. However the accurate and robust measurement of microRNAs in biofluids is made challenging by a number of factors. Firstly the microRNAs themselves are very short and consist of highly divergent sequences with large variation in GC content. This variability leads to very different hybridization properties between different microRNA sequences and makes simultaneous measurement of all microRNAs challenging. Although microRNAs are highly divergent in general, individual members of microRNA families display high sequence similarity, sometimes varying only by a single nucleotide making proper differentiation challenging. Secondly, the overall amount of microRNA, and RNA in general, that is present in samples such as serum/plasma and urine is very low. The use of a highly sensitive, specific and accurate microRNA qPCR method that allows linear detection of microRNAs even at very low target concentrations is therefore essential.

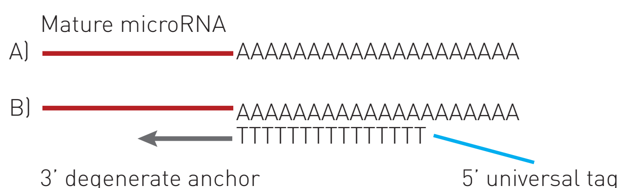
LNA™-enhanced qPCR for microRNA profiling in biofluids

Several different methods for real-time quantification of microRNAs are currently available. Gene specific reverse transcription (RT) can add sensitivity and specificity, but leads to increased sample requirement and complicated experimental procedures. A universal RT step allows hundreds of targets to be quantified from the same cDNA synthesis, leading to dramatically decreased sample requirements and increased ease of use. However, microRNA amplification using DNA-based primers has restricted sensitivity, especially for AT-rich targets, as well as difficulties with single nucleotide mismatches.

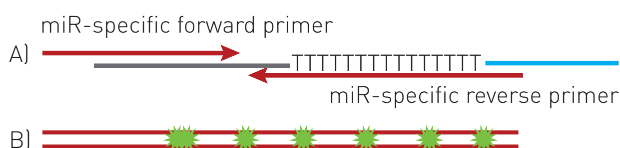
We have developed a method for microRNA qPCR in which a single cDNA synthesis reaction is followed by PCR amplification using two microRNA-specific, LNA™-enhanced primers (figure 1). The inclusion of LNA™ in the PCR primers allows

Figure 1. Schematic outline of the miRCURY LNA™ Universal RT microRNA PCR System. A polyA tail is added to the mature microRNA template (step 1A). cDNA is synthesized using a PolyT primer with a 3' degenerate anchor and a 5' universal tag (step 1B).The cDNA template is then amplified using microRNA-specific and LNA™-enhanced forward and reverse primers (step 2A). SYBR® Green is used for detection (step 2B).

Step 1: First-strand synthesis (RT)



Step 2: Real-time PCR amplification



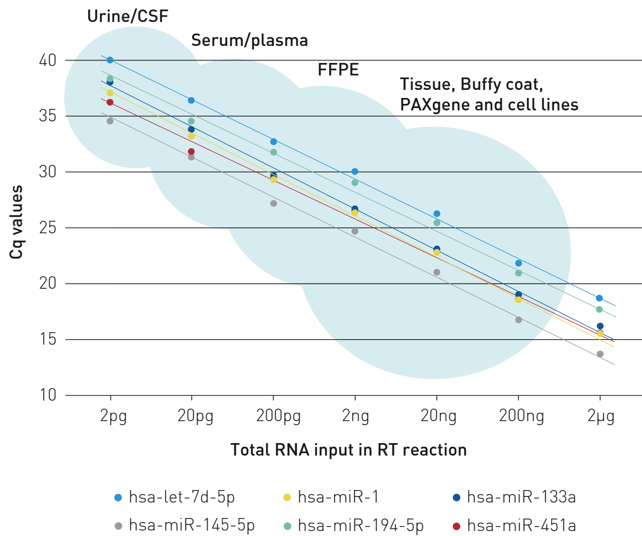
the design of short, yet specific, high affinity primers even for AT rich sequences [5].

The unique combination of two microRNA-specific primers with highly optimized SYBR® Green PCR master mix means that the system retains specificity at the same time as being extremely sensitive (figure 2). Profiling of up to 378 microRNAs is possible from as little as 20ng total RNA or the equivalent of 20µl biofluids such as serum/plasma or urine without the need for pre-amplification. This not only limits the number of handling steps but also avoids the risk of introducing artificial bias and thereby limiting misleading false positive amplification results.

The miRCURY LNA™ Universal RT microRNA PCR system combines wet-lab validated microRNA qPCR assays in ready-to-use PCR plates allowing highly accurate and robust microRNA profiling using readily available instrumentation. The optimized and simple workflow is easily adapted to high-throughput applications and well-suited for a clinical automatable workflow, fully compatible with standard FDA-approved qPCR equipment.

Thousands of biofluid samples have been profiled, including blood derived serum/plasma as well as urine, to accurately determine normal reference ranges for circulating microRNAs. Procedures have been developed for sample and data quality

Figure 2. Superior sensitivity and linearity of the LNA™-enhanced microRNA qPCR platform. All microRNA primers sets are thoroughly wet-lab validated ensuring accurate and reliable microRNA detection. Data from the amplification of 6 microRNAs in serial dilutions of human reference RNA are shown. All microRNA assays exhibit linear read-out with correlation coefficients R2 > 0,99.



control to secure technical excellence and reveal any unwanted bias resulting from pre-analytical variables (13).

Based on our extensive experience with microRNA profiling in biofluids we have created a Toxicology Focus microRNA PCR panel for biomarker discovery in human, rat, monkey or dog. The assays have been preselected based on literature as well as from empirical data from thousands of Exiqon's in-house studies of microRNA expression profiling in clinical and biological samples. The panels contain organ specific microRNA markers for toxicity induced damage, tissue specific microRNAs and microRNAs highly expressed in many tissues and biofluids including reference gene candidates for circulating microRNAs. Here we report results of a toxicology study demonstrating the discovery of microRNA biomarkers for monitoring the effect of a potent reference nephrotoxin that is known to cause acute renal failure through induction of proximal tubular damage. The overall levels of microRNAs in urine are usually increased with any pathophysiological condition compared to normal controls, creating a challenge in terms of data pre-processing and normalization. Here we suggest a normalization procedure consisting of creatinine-based normalization of input volume and microRNA reference genes for calculation of relative differences between samples. Using this procedure we can identify a number of microRNAs as potential markers for kidney damage.

Materials and Methods

Experimental protocol

Han-Wistar rats (male / 6 weeks old / 4 per group) were treated intraperitoneally with a single toxic dose of the reference nephrotoxin or with physiological saline as a control. Urine samples were collected over-night into cooled containers on day 3 and 5 post nephrotoxin administration without use of any further stabilizing buffer. Rats were also sacrificed at day 3 and 5 for histopathological evaluation.

RNA Extraction

Upon collection, the urine was centrifuged to remove debris and food spill and stored at -20 until analysis. The urine was thawed on ice and centrifuged at 3000 x g for 5 min in a microcentrifuge at +4°C. 200 µl urine supernatant per sample was used as starting material for the RNA isolation. RNA was extracted following Exiqon's recommended protocol for RNA isolation from Biofluids adding 1 µg MS2 RNA per sample as carrier to the lysis buffer. The RNA was eluted with 50 µl RNase free water and stored at -80°C prior to use.

microRNA qPCR

microRNA qPCR was performed according to the protocol for the miRCURY LNA™ Universal RT microRNA PCR System. Briefly, 4 µl RNA was reverse transcribed in 20 µl reactions using the miRCURY LNA™ Universal cDNA synthesis kit (Exiqon). cDNA was diluted 50 x and 5 µl used in 10 µl PCR reactions with miRCURY LNA™ SYBR® Green master Mix (Exiqon) on the Toxicology Focus microRNA PCR Panel. Negative controls with 1 µg MS2 carrier RNA as mock template from the reverse transcription reaction were performed and profiled like the samples. The amplification was performed in a LightCycler® 480 Real-Time PCR System (Roche). The amplification curves were analyzed using the Roche LC software, both for determination of the quantification cycle Cq (by the 2nd max derivative method) and for melting curve analysis.

Data Analysis, Normalization and Statistics

The amplification efficiency was calculated using an adaption of the LinReg algorithm. All assays were inspected for distinct melting curves and the Tm was checked to be within known specifications for the assay. Only assays with Cq values below 37 and that differed from their corresponding negative control with more than 4.8 Cq values were included in the analysis.

In order to equalize any diuretic dilution effects, data were normalized for creatinine concentration (8). Creatinine in urine samples was measured with a clinical pathology analyzer using standard protocols. Log2 data (Cqs) were converted into a linear scale assuming amplification efficiency of 2 for every

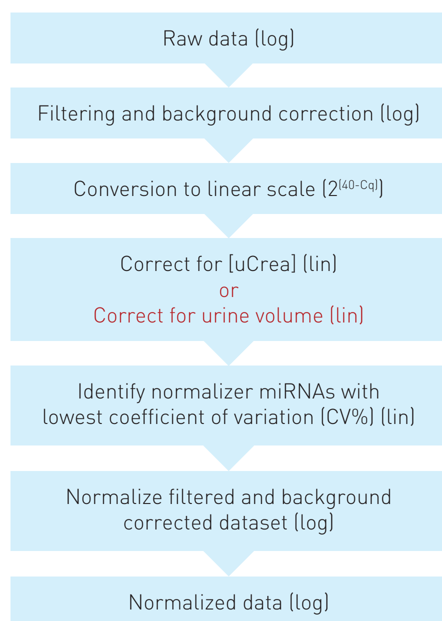


Figure 3. Schematic overview of the data normalization procedure. The raw Cq values were filtered and background corrected. After conversion into linear scale the data were divided through their relative creatinine correction factor. The corrected linear dataset was then analyzed for the microRNAs showing lowest coefficient of variation. The average Cq values of these microRNA provided a sample specific normalizer deviation factor that was used to normalize the filtered and background corrected dataset.

assay and all samples ($2^{(40-Cq)}$). The data were corrected for creatinine amount uCrea [mmol/l] by dividing Cq values with amount of uCrea per subject and subsequently standard deviation, average and coefficient of variation (CV%) was calculated for each microRNA across the samples. Based on this, 4 microRNAs that showed lowest CV% were selected. The average of these 4 normalizers (as log2) from the original data was used for normalization: To deal with the necessity of using a fixed value for assays that gave no signal for a particular datapoint, the data were kept on the original scale of log2 (40 to 1) to calculate how much each sample deviates from the overall normalizers' average value. For each sample (i) we calculated the observed deviation from its four normalizers' average towards the overall normalizers' average across all samples (n):

$$\Delta Cq4N_{(sample\ i)} = Cq4N_{(sample\ i)} - 1/n * \left(_{(i=1 \rightarrow n)} \sum Cq4N_{(sample\ i)} \right)$$

Then for each sample the measured Cq value for each microRNA ($Cq_{(sample\ i)}$) was corrected for this normalizer deviation factor($\Delta Cq4N$) to obtain a normalized Cq (nCq):

$$nCq_{(sample\ i)} = Cq_{(sample\ i)} - \Delta Cq4N_{(sample\ i)}$$

Then the Cq of assays that gave no signal was deliberately set to 40 thus allowing these assays to be included in the in further calculations despite not being detected. In order to be taken into account in the analysis, a maximum of 4 data points per microRNA assay were allowed to have a Cq of 40.

The normalized Cq values of the microRNAs expressed in all samples were used in the unsupervised two-way hierarchical clustering of microRNAs and in the Principal Component Analysis (PCA). Student t-tests were used to test the significance of the changes between controls and treated at each time point. The significance data were further corrected for multiple testing parameters (Bonferroni-corrected). Data preprocessing and statistics were done with Exiqon GenEx qPCR data analysis software (Exiqon) with Exiqon qPCR Plate Import Wizard and Exiqon Data Analysis Guide.

Results

In order to identify microRNA biomarkers for nephrotoxicity, we treated Han-Wistar rats with a reference nephrotoxin or physiological saline and collected urine samples after three and five days. Both treated and control rats were subjected to histopathological evaluation for kidney necrosis and an overview of the sample types and results can be seen in table 1. RNA was isolated from the urine samples with the addition of carrier RNA for optimal yield and reproducibility [6] and subjected to qPCR profiling on the Toxicology Focus microRNA PCR Panel. The resulting data were pre-processed and normalized according to the procedure described in the material and methods section (figure 3).

Table 1. Overview of the experimental design and level of kidney necrosis. Urine was collected on day 3 and 5 post treatment from 4 rats per group. Histopathological examination confirmed proximal tubule necrosis in the kidneys for the nephrotoxin treated animals.

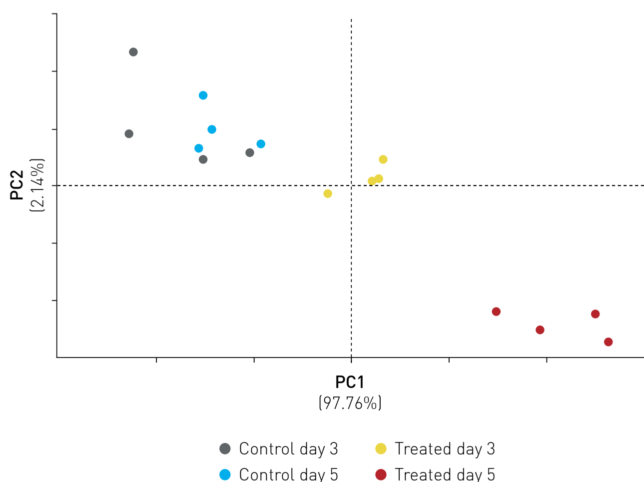
Time	Day 3		Day 5	
Treatment	Control	Treated	Control	Treated
Number per group	N=4	N=4	N=4	N=4
Kidney Necrosis	None	Mild	None	Moderate

In total, 35 microRNAs passed QC and cut-off criteria. These microRNAs were included in the subsequent data analysis.

Unsupervised clustering analysis demonstrates that the microRNA profile changes significantly upon reference nephrotoxin administration and that the differences between the control and treated samples increases over time (figure 4 and 5). Principal Component Analysis (PCA) illustrates that the samples separate in 3 distinct groups. The groups also correspond to the level of kidney necrosis thereby underlining that the biological differences between the samples are the principal components differentiating the samples. A two-way unsupervised hierarchical clustering of the 35 microRNAs and samples confirms that the control and treated samples cluster separately. The treated samples are further subdivided according to sampling time.

A subsequent supervised analysis revealed a subset of 18 microRNAs showing a significantly higher expression signal on day 5 compared to the corresponding control groups (CI: 99%, data not shown). One of these assays (rno-miR-378-3p) alsorevealed a significant change at day 3 (CI: 99%, equaling a Bonferroni corrected p value of 0.00029). The 18 microRNAs that were significantly enriched in urine after reference nephrotoxin administration are listed in table 2 with their corresponding p-values. Of these, 14 microRNAs are known to be expressed in kidney according to smirnaDB (ref: http://www.mirz.unibas.ch/cloningprofiles/). Detailed fold changes for microRNAs rno-miR-21-5p, rno-miR-34a-5p and rno-miR-192-5p are shown in figure 6.

Figure 4. Clear separation of treatment and control groups using Principal Component Analysis. The principal component analysis was performed using normalized Cq values from the 35 microRNAs that passed the QC and cutoff criteria in all samples. Clear clustering of control versus treatment groups is seen, as well as distinct differential grouping of treatment day 3 versus treatment day 5. No outliers were detected.



Discussion

We have illustrated how the use of Exiqon's Toxicology Focus microRNA PCR Panel enables reliable and accurate monitoring of nephrotoxicity in a rat animal model. Dramatic changes in microRNA expression levels in urine are detected after administration of a reference nephrotoxin. Both the PCA plot and the Heat map expression profiles displayed clear distinction between treated and untreated animals and a clear correlation between tissue damage and microRNA detection levels in urine.

Out of 35 microRNAs that passed the QC and cut-off criteria, 18 showed significant differential expression between control and treated samples at day 5 p.a. demonstrating that the assays included in the panel are highly relevant for the investigation of the effects of organ damage in urine samples.

Table 2. microRNAs showing statistically significant increased levels in urine following treatment. Table listing microRNAs that are significantly enriched in urine after reference nephrotoxin administration and their corresponding p-values. microRNAs listed in bold are known to be expressed in kidney (smiRNADB).

microRNA	P-value	microRNA	P-value
rno-miR-328a-3p	7.50E-08	rno-miR-151-5p	7.49E-05
rno-miR-30c-5p	4.97E-06	**rno-miR-22-3p**	9.22E-05
rno-miR-378-3p	1.54E-05	**rno-miR-29a-3p**	0.0001032
rno-let-7b-5p	1.87E-05	**rno-let-7c-5p**	0.0001433
rno-miR-375-3p	2.13E-05	rno-miR-320-3p	0.0001632
rno-miR-16-5p	2.29E-05	**rno-miR-192-5p**	0.0001787
rno-miR-15b-5p	4.60E-05	**rno-let-7a-5p**	0.0001867
rno-miR-26b-5p	5.03E-05	**rno-miR-21-5p**	0.0002313
rno-miR-29c-3p	7.27E-05	**rno-miR-34a-5p**	0.0002817

Of the microRNAs that showed significant changes, over 75% are known to be expressed in kidney, indicating that the developing organ necrosis is causing these microRNAs to be released into the urine. A single microRNA (rno-miR-378-3p) also showed significant changes at three days after nephrotoxin administration. This could signify that this microRNA might be a candidate for early detection of kidney damage.

The selected microRNAs (rno-miR-21-5p, rno-miR-34a-5p, and rno-miR-192-5p) shown in figure 6 have also been identified in other studies of acute kidney injury or renal toxicology.

Increased expression of several microRNAs, including miR21-5p and miR-192-5p has previously been linked to renal fibrosis

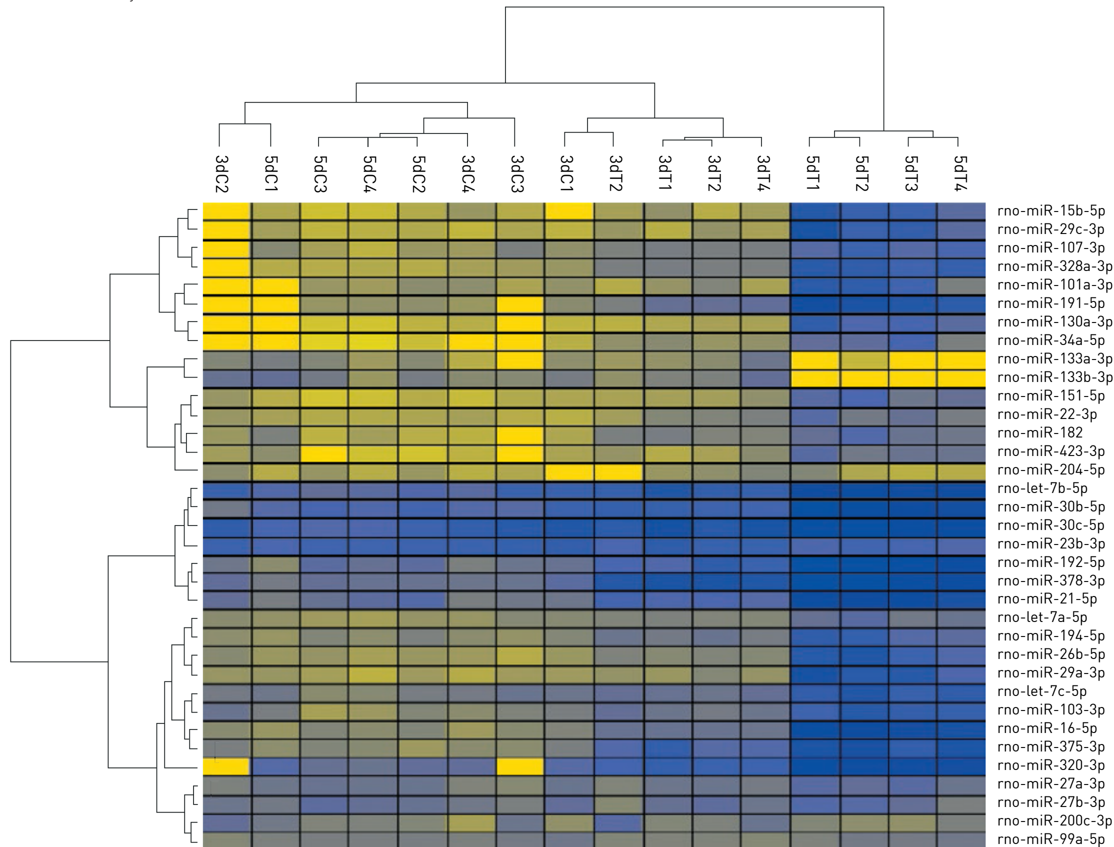


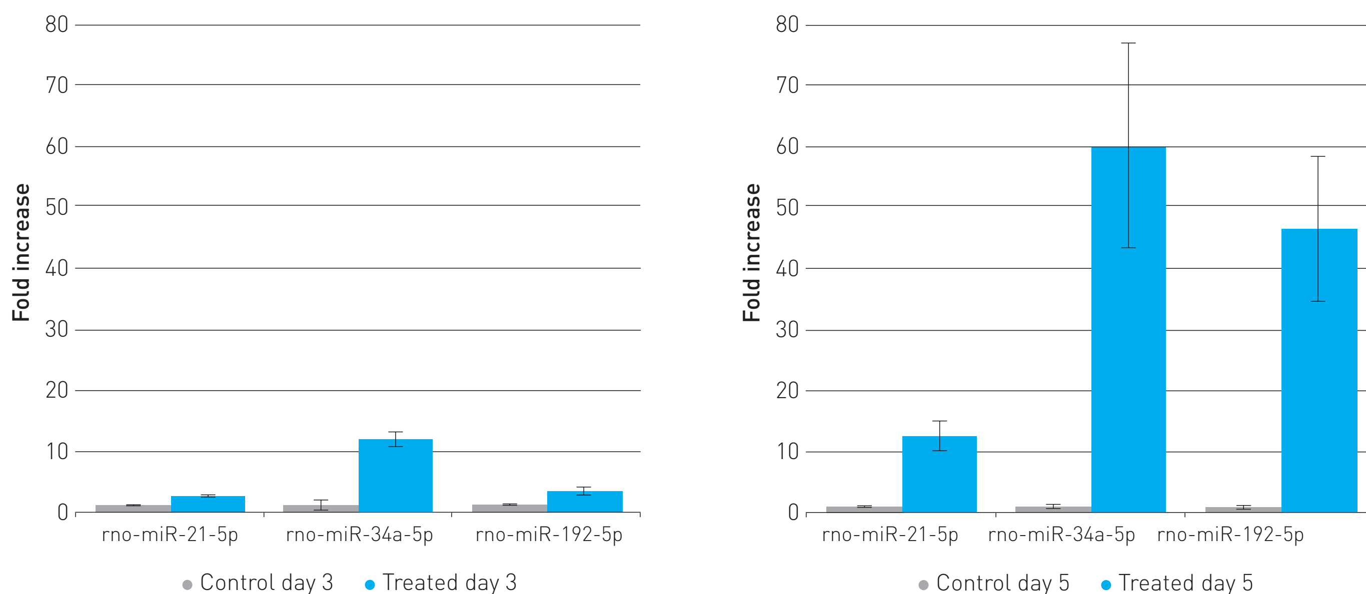
Figure 5. Heat Map displaying differential microRNA expression in control versus treated animals. Unsupervised clustering analysis was performed was performed using normalized Cq values from the 35 microRNAs that passed the QC and cutoff criteria in all samples using Wards algorithm. The color scale illustrates the relative expression level of a microRNA across all samples: blue color represents an expression level above average, yellow color represents an expression level lower than the average. Samples are as follows: 3dC1-4 = Controls after 3 days; 3dT1-4= Treated after 3 days; 5dC1-4 = Controls after 5 days; 5dT1-4= Treated after 5 days)

in rats or mice [9,10,11]. In another nephrotoxicity study, Bhatt et al. showed that p53 induced upregulation of miR-34a in mouse kidney tissue was associated with renal cell survival [12].

We have also demonstrated a suitable data processing and normalization strategy for this type of microRNA profiling study. In order to ensure reliable data, we used data from the negative control to filter the results. This resulted in a set of 35 microRNAs considered reliably detected in the study. As the level of RNA isolated from urine samples is very low, a carrier RNA was used during the purification to ensure consistent and reproducible yields. This meant that the measurement of endogenous RNA levels for input normalization was not possible. In order to normalize input amounts we investigated the use of total urine volume (here 24h) or creatinine levels. Both methods gave comparable results, however, creatinine normalization is also applicable even when total urine amounts are unknown. The creatinine normalized data were further analyzed to find the microRNAs with the lowest coefficient of variation (CV%) across the dataset to be used as normalizers. Four different microRNA each from a different transcription unit or cluster were selected (rno-miR-27a-3p, rno-miR-200c-3p, rno-miR-23b-3p, rno-miR-99a-5p). The selection from 4 different localization clusters aims to minimize the risk that the selected microRNAs are intrinsically co-regulated and should ensure that their transcription and expression levels are as independent from each other as possible. The limited number of microRNAs and the large differences in microRNA levels between samples prohibited use of a global mean normalization strategy.

The differentially regulated microRNAs discovered in this study may well be regarded as potential biomarkers allowing the detection of kidney damage in urine. Future studies to validate these potential biomarkers could be performed on increased sample numbers with the specific subset of microRNAs (including reference assays), using custom designed Pick-&-Mix microRNA PCR panels.

Figure 6. Fold increase of rno-miR-21-5p, rno miR-34a-5p and rno-miR-192 -5p upon reference nephrotoxin treatment. The normalized Cq values for each microRNA were transformed to linear scale expression values ($2^{(40-nCq)}$) and divided through the average of the control group of the respective day. The gray bars show the mean expression for the controls (+/- SE) for each control group (day 3 and 5) while the blue bars show the mean fold increase of expression for each reference nephrotoxin treated group (+/- SE) (day 3 and 5) based on their corresponding controls.



microRNA profiling in biofluid samples is challenging and the development of robust biomarkers requires not only a highly sensitive and accurate microRNA detection method, but also optimized and standardized procedures for sample handling and preparation as well as reliable methods for sample and data quality control.

Exiqon has many years of experience with both microRNA profiling and biomarker discovery and validation in biofluid samples.

Visit our Biofluids reading room for more information as well as recommendations and free guidelines: **www.exiqon.com/biofluids**

Acknowledgements

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Ordering information

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