# microRNA biomarker discovery for asthma in a mouse model

Katrin Milger[1], Stefan Dehmel[1], Susanne Krauss-Etschmann[1], Kim Bundvig Barken[2], Ina K Dahlsveen[2] and Peter Mouritzen[2]
1. Comprehensive Pneumology Center, LMU, Munich, Germany; 2. Exiqon A/S, Vedbæk, Denmark

In order to identify potential microRNA biomarkers for asthma, a well described mouse model for asthmatic response was used for microRNA profiling. RNA from the equivalent of 1µL serum was enough to profile over 120 mouse microRNAs on the highly sensitive miRCURY LNA™ Serum/Plasma microRNA Focus Panels. Several potential microRNA biomarkers were identified that showed differential expression between the experimental and control groups. This study indicates that microRNAs are putative candidates for use in the diagnosis of this very common respiratory disease

## Introduction

Asthma is a common chronic inflammatory disorder of the airways and is believed to affect more than 300 million people worldwide. It is a heterogeneous disease with several different phenotypes, generally triggered by a combination of environmental and genetic factors. Diagnosis usually depends on pulmonary function tests and can be difficult in young children, since the symptoms resemble viral infections of the respiratory tract. However, early asthma diagnosis is important to initiate appropriate treatment.

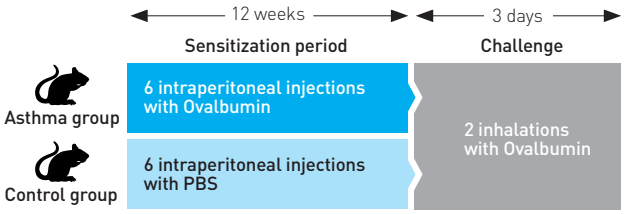
microRNAs are small regulating RNAs that have been shown to play important roles in most cellular and developmental processes and have been implicated in a large number of human diseases. Due to their wide-ranging biological potential and the fact that microRNAs seem to be relatively stable in readily available clinical samples, these small 20-22 nt molecules are prime candidates for use as minimally invasive biomarkers in molecular diagnostics. microRNA biomarkers in serum might have the potential to confirm asthma diagnosis as well as to indicate disease phenotype.

Profiling microRNA in biofluids such as serum is particularly challenging due to the limited material available. Here we demonstrate that the highly sensitive and accurate miRCURY LNA™ Universal RT microRNA PCR system allows microRNA profiling from the equivalent of just 1µL serum. The profiling was performed in ready-to-use PCR panels using the new LightCycler® 96 System from Roche for the optimal combination of ease-of-use and high performance.

## Experimental design and methods

We used a well characterized and widely used experimental mouse model to test if microRNA biomarkers could be identified for asthma. One group of mice were systemically sensitized by fortnightly intraperitoneal ovalbumin + alum injections over a period of 12 weeks, while a second control group received PBS injections. Both groups of mice were then challenged twice by inhalation of aerolized ovalbumin (Figure 1).

**Figure 1. Ovalbumin model of experimental asthma.** Each group contained 6 BALB/c mice. All samples were collected 1 day after the last ovalbumin inhalation.




The mice were sacrificed 1 day after the last aerosol inhalation and blood, lung and BAL were stored for further analyses. Analyses including serum-Ig-E-levels, lung histology and cell differentiation in BAL were performed and confirmed the asthma phenotype. Before isolating RNA, all serum samples were investigated for hemolysis by measuring the absorption at 415nm to estimate the amount of hemoglobin released from red blood cells. For each mouse, RNA from 50µL serum was isolated using Exiqon's recommended procedure and eluted in 50µL of water.

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Figure 2. From samples to analyzed data in four steps.

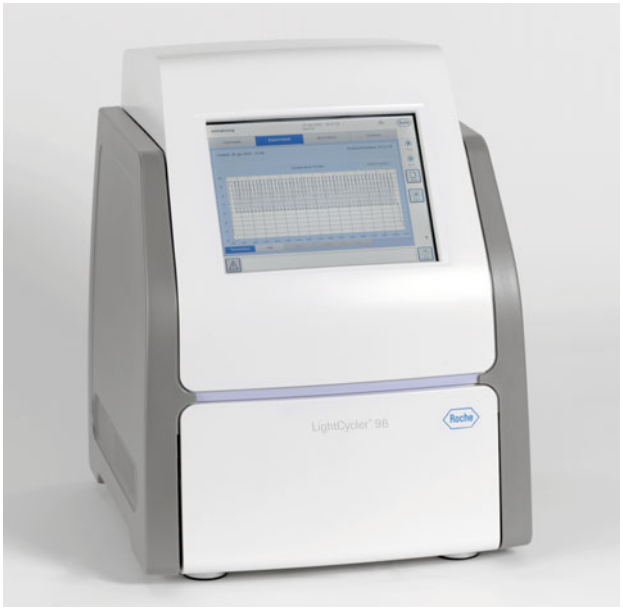
**1. Sample:** Isolate RNA from serum

**2. QC:** Optimize RNA input to cDNA synthesis and check for hemolysis

**3. Profile:** Profile samples on serum/plasma focus panels

**4. Analyze:** Collect and analyze qPCR data using GenEx

Figure 3. The Roche LightCycler® 96 was used for quantitative real-time PCR.




To optimize RNA input and to check for qPCR inhibitors, one sample from each group was used to perform reverse transcription reactions with different RNA inputs (1, 2 and 4 µL) and tested using Exiqon's recommended set of control assays.

For microRNA profiling, 1µL eluted RNA equivalent to 1µL serum was used as template in a 20µL Universal cDNA reaction. RNA from the 6 mice in each group was analyzed using Exiqon's 96well Serum/Plasma Focus microRNA PCR Panels, with the miRCURY LNA™ Universal cDNA Synthesis and SYBR® Green Master Mix kits. The amplification was performed in a LightCycler® 96 System. Data were preprocessed and analyzed in the GenEx Software from Exiqon designed for fast and easy qPCR data import and analysis. The ΔCq (mmu-miR-23a – mmu-miR-451) was evaluated in order to confirm that none of the samples were affected by hemolysis (all samples had ΔCq values below 3).

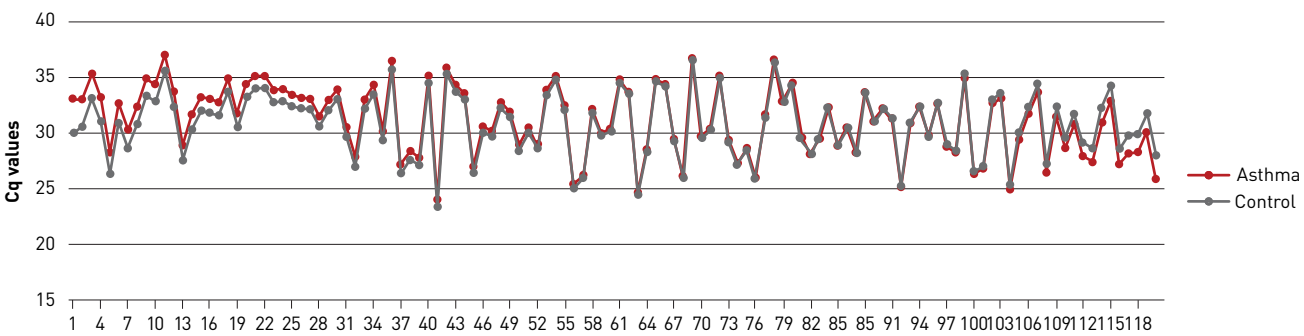
GeNorm was used to identify useful reference assays for normalization. Mmu-miR-93 and mmu-miR-423-3p were both found to be stably expressed across sample groups and were thus used for normalization.

## Results

The Serum/Plasma Focus microRNA PCR panel contains 131 assays that are annotated for mouse (the total number of assays is 179). From the equivalent of 1µL serum, 121 microRNAs out of the 131 murine assays were detected with a Cq value of 37 or less, giving a call rate of 92%.

Figure 4 shows a comparison of the average microRNA profiles from the two sample groups illustrating how expression levels of most of the microRNAs are very similar between the asthma and control samples.

**Figure 4. Average microRNA expression profiles from the equivalent of 1µL serum.** Average Cq values for the 121 microRNAs detected in each experimental group are shown. microRNAs are sorted by ΔCq between the asthma and control group.

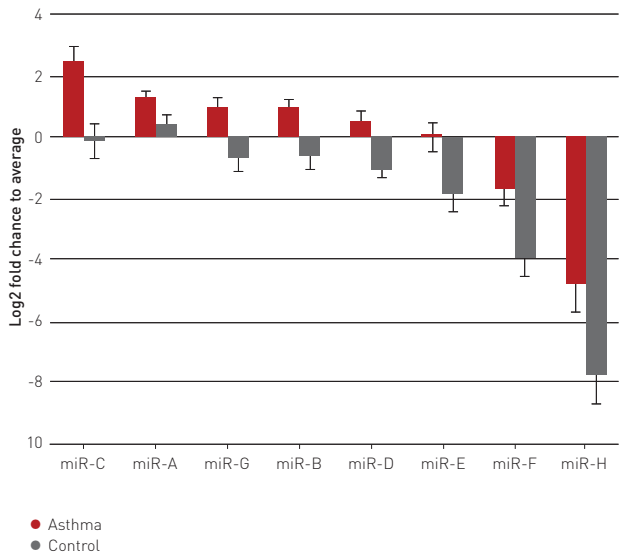


Out of the 121 microRNAs detected, 27 were found to be differentially expressed with a P-value below 0.05. However, when performing multiple testing, the threshold value should be less than $4.00 \times 10^{-4}$ according to Dunn-Bonferroni correction, which reduces the number of significant differentially expressed microRNAs to 8 (Table 1 and Figure 5). Several of these differentially expressed microRNAs have previously been reported in the literature as being deregulated in lung samples of asthmatics or animal models of asthma.

**Table 1. Differentially expressed microRNAs.** The log 2 fold change and P-values for the 8 differentially expressed microRNAs with statistical significance according to the Dunn-Bonferroni correction are listed.

| (Asthma) vs (Control) | Fold change (log2) | P-Value |
|---|---|---|
| miR-A | 0.85792 | $3.18 \times 10^{-4}$ |
| miR-B | 1.65292 | $1.91 \times 10^{-5}$ |
| miR-C | 2.48708 | $7.18 \times 10^{-6}$ |
| miR-D | 1.58625 | $4.48 \times 10^{-6}$ |
| miR-E | 2.03292 | $1.67 \times 10^{-5}$ |
| miR-F | 2.18208 | $2.70 \times 10^{-5}$ |
| miR-G | 1.81792 | $7.35 \times 10^{-6}$ |
| miR-H | 2.86292 | $2.08 \times 10^{-4}$ |

**Figure 5. microRNAs with significant differential expression in mice with an asthmatic response compared to control group.** Normalized log 2 fold changes from average signal of all assays/samples for the 6 replicates of mice with asthmatic response (red) and controls (gray).Standard deviation between the 6 replicates is shown. P values are all below $4.00 \times 10^{-4}$.

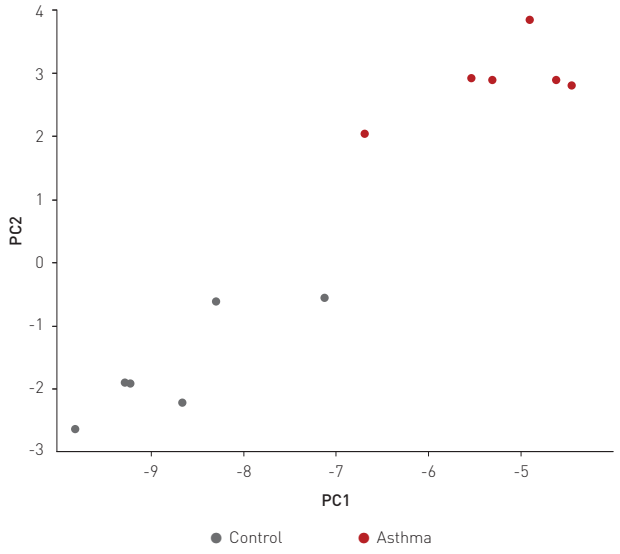



The top-8 differentially expressed microRNAs were used to perform a Principal Component Analysis (PCA, Figure 6) which clearly illustrates how the mice with the asthmatic response separate from the control group. This indicates that the set of 8 microRNAs might be useful as a biomarker signature for asthmatic response.

We have demonstrated that the miRCURY LNA™ Universal RT microRNA PCR system is sensitive enough to enable reliable microRNA profiling and to identify potential biomarkers from the equivalent of as little as 1µL mouse serum. Although the number of individuals in each sample group was relatively low, we could also identify several microRNAs with statistically significant differences between the experimental group, demonstrating the accuracy of the system.

The identified microRNAs could represent putative biomarkers for asthma and further validation in other model systems can be performed on this subset of microRNAs using Exiqon's Pick-&-Mix microRNA PCR Panels for fully customized PCR panel design.

**Figure 6. Principal component analysis showing clear separation of the two experimental groups.** The top 8 differentially expressed microRNAs were used to generate the PCA plot. Mice with asthmatic response (red) and control mice (gray) clearly clusters separately from each other.




For detailed recommendations of how to perform microRNA profiling in serum and other biofluids, please download our guidelines from **www.exiqon.com/biofluids**

## Ordering information

| Reagents | Product description | Product no. |
|---|---|---|
| Universal cDNA Synthesis Kit II | Polyadenylation and cDNA synthesis kit (8 to 64 rxns) | 203301 |
| ExiLENT SYBR® Green master mix, 2.5ml | 250 rxns of 20µl or 500 rxns of 10µl | 203402 |
| ExiLENT SYBR® Green master mix, 20ml | 2000 rxns of 20µl or 4000 rxns of 10µl | 203420 |
| **miRNome panels** | **Product description** | **Product no.** |
| microRNA Ready-to-Use PCR, Human panel I+II, V3 | 4x panel I and 4x panel II in 384-well PCR plates, 752 human microRNAs, control and reference assays* | 203611 203612 |
| microRNA Ready-to-Use PCR, Human panel I, V3 | 4x panel I in 384-well PCR plates, 372 human microRNAs, control and reference assays* | 203613 203614 |
| microRNA Ready- to-Use PCR, Mouse & Rat panel I+II, V3 | 4x panel I and 4x panel II in 384-well PCR plates, 752 mouse and rat microRNAs, control and reference assays* | 203709 203710 |
| microRNA Ready-to-Use PCR, Mouse & Rat panel I, V3 | 4x panel I in 384-well PCR plates, 372 mouse and rat microRNAs, control and reference assays* | 203711 203712 |
| **Focus microRNA PCR Panel** | **Product description** | **Product no.** |
| Serum/Plasma Focus , V3, 96 well Ready-to-Use plates | 4 Panels in 8 PCR plates. Each panel contains LNA™ primers for 179 human serum/plasma microRNAs, control and reference assays* | 203836-203839 |
| Serum/Plasma Focus , V3, 384 well Ready-to-Use plates | 8 panels in 2 PCR plates. Each panel contains LNA™ primers for 179 human serum/plasma microRNAs, control and reference assays* | 203842 203843 |
| Cancer Focus , V3, 96 well Ready-to-Use plates | 4 Panels in 4 PCR plates. Each panel contains LNA™ primers for 84 human cancer microRNAs, control and reference assays* | 203832-203835 |
| Cancer Focus , V3, 384 well Ready-to-Use plates | 8 Panels supplied in two PCR plates. Each panel contains LNA™ primers for 84 human cancer microRNAs, control and reference assays* | 203840 203841 |
| microRNA QC PCR Panel, 96 well Ready-to-Use plates | 16 Ready-to-Use microRNA QC PCR Panels, supplied in two 96-well plates. Each panel comprises 6 LNA™ microRNA primer sets and 5 reference genes and UniSp3 for evaluation of microRNA quality.* | 203844-203847 |
| microRNA QC PCR Panel, 384 well Ready-to-Use plates | 32 Ready-to-Use microRNA QC PCR Panels, supplied in one 384-well plate. Each panel comprises 6 LNA™ microRNA primer sets and 5 reference genes and UniSp3 for evaluation of microRNA quality.* | 203848 203849 |

| Individual assays | Product description | Product no. |
|---|---|---|
| xxx-miR-xxx, LNA™ PCR primer set, UniRT | microRNA primer set, 200 rxns | 204000-206997 |
| Reference gene PCR primer set, UniRT | Reference gene primer set, 200 rxns | 203901-203912 |
| **Pick-&-Mix microRNA PCR panel** | **Product description** | **Product no.** |
| 96 well, Ready-to-Use plates | 8 PCR plates with custom selection of microRNA primer sets* | 203801 |
| 384 well, Ready-to-Use plates | 8 PCR plates with custom selection of microRNA primer sets* | 203802 |
| **RNA Spike-In** | **Product description** | **Product no.** |
| RNA Spike-in kit, UniRT | miRCURY LNA™ Universal RT microRNA PCR, Set of two vials with synthetic RNA spike-in templates for qPCR control (UniSp2, UniSp4, UniSp5 RNA Spike-in template mix and cel-miR-39-3p RNA Spike-in template) | 203203 |
| UniSp2, LNA™ control primer set, UniRT | miRCURY LNA™ Universal RT microRNA PCR, spike-in control primer set, 200 rxns | 203950 |
| UniSp5, LNA™ control primer set, UniRT | miRCURY LNA™ Universal RT microRNA PCR, spike-in control primer set, 200 rxns | 203951 |
| cel-miR-39-3p, LNA™ control primer set, UniRT | miRCURY LNA™ Universal RT microRNA PCR, spike-in control primer set, 200 rxns | 203952 |
| UniSp4, LNA™ control primer set, UniRT | miRCURY LNA™ Universal RT microRNA PCR, spike-in control primer set, 200 rxns | 203953 |
| **Exiqon GenEx Software** | **Product description** | **Product no.** |
| Pro Industrial | Exiqon GenEx, qPCR analysis software, industrial license | 207005 |
| Pro Academic | Exiqon GenEx, qPCR analysis software, academic license | 207006 |
| Enterprise Industrial | Exiqon GenEx, qPCR analysis software, industrial license | 207007 |
| Enterprise Academic | Exiqon GenEx, qPCR analysis software, academic license | 207008 |
| Import wizard | Exiqon qPCR plate import wizard addition to GenEx, for current GenEx customers | 207009 |
| Pro, Portable USB license key | Multiple-user license upgrade to the Pro version | 207011 |
| Enterprise, Portable USB license key | Multiple-user license upgrade to the Enterprise version | 207012 |

* Each panel product is available in different types of plates for specific real-time PCR instrument compatibility. Plate types for all major brands of real-time PCR instruments are covered including ABI (7000 series, FAST series, StepOnePlus, 7900HT and Viia 7), Roche (LightCycler 480), BioRad (iCycler, iQ series, and CFX384), Eppendorf (Mastercycler ep Realplex), and Stratagene (Mx4000 and Mx3000 series). The product number relates to the specific plate type. For details, please visit www.exiqon.com/mirna-pcr.

## Contact information


**Outside North America**

Phone: +45 45 65 09 29 · Fax: +45 45 66 18 88

**North America**

Phone: +1 781 376 4150 · Fax: +1 781 376 4152

**www.exiqon.com/contact**





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