



## Review Article

## Improved microRNA quantification in total RNA from clinical samples

Ditte Andreassen<sup>1</sup>, Jacob U. Fog<sup>1</sup>, William Biggs, Jesper Salomon, Ina K. Dahlsveen\*, Adam Baker, Peter Mouritzen

Exiqon A/S, Skelstedet 16, DK-2950 Vedbaek, Denmark

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## ABSTRACT

microRNAs are small regulatory RNAs that are currently emerging as new biomarkers for cancer and other diseases. In order for biomarkers to be useful in clinical settings, they should be accurately and reliably detected in clinical samples such as formalin fixed paraffin embedded (FFPE) sections and blood serum or plasma. These types of samples represent a challenge in terms of microRNA quantification. A newly developed method for microRNA qPCR using Locked Nucleic Acid (LNA<sup>TM</sup>)-enhanced primers enables accurate and reproducible quantification of microRNAs in scarce clinical samples. Here we show that LNA<sup>TM</sup>-based microRNA qPCR enables biomarker screening using very low amounts of total RNA from FFPE samples and the results are compared to microarray analysis data. We also present evidence that the addition of a small carrier RNA prior to total RNA extraction, improves microRNA quantification in blood plasma and laser capture microdissected (LCM) sections of FFPE samples.

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## 1. Introduction

## 1.1. microRNAs are promising biomarkers

microRNAs comprise a family of highly conserved small non-coding RNAs (~22 nt). As regulators of post-transcriptional gene expression, microRNAs play an essential role in a large number of biological and pathological processes. It is now established that altered microRNA expression profiles are associated with a number of different diseases including heart disease, neurological disorders and human cancers [1–3]. This has suggested the use of microRNAs as biomarkers for disease diagnosis and prognosis. A number of recent reports have confirmed the usefulness of microRNAs as biomarkers in cancer [4,5].

As microRNAs are more stable than mRNAs, they are good candidates for use as biomarkers [6]. However, due to their short length and the high sequence similarity within microRNA families, reliable and accurate quantification is still a challenge. A valuable biomarker requires robust and reproducible assays that work in clinically available samples as well as archived material. With the advent of more complete screening protocols where precious clinical samples are being analyzed for a variety of biomarkers, the importance of reduced sample requirements is increasing, generating a need for extremely sensitive assays.

The most commonly available archived material often used for biomarker discovery and validation is FFPE tissue [7]. However, to-

tal RNA isolated from FFPE material represents a challenge due the fact that formalin fixation causes cross links and degradation that result in isolation of short degraded RNA fragments. Formalin also causes chemical modifications that negatively affect qPCR efficiency [8]. This results in poor RNA yields and inferior qPCR performance. In addition, variation between separate RNA extractions from FFPE samples is significant.

Blood serum and plasma also represent important sample types for investigating microRNAs as biomarkers [9,10]. The nucleic acids present in carefully prepared serum and plasma are presumed to be extracellular. The level of microRNAs in such samples is very low and efficient and reproducible recovery of this RNA is problematic. In addition, RNA purified from plasma can also contain inhibitors that affect qPCR efficiency. Taken together, these issues generate a great need for reproducible RNA isolation and miRNA quantification methods that are extremely sensitive and robust.

## 1.2. A unique method for microRNA quantification

Several different methods for real-time quantification of microRNAs have been developed attempting to achieve the optimal combination of sensitivity and specificity [11]. 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 leads to restricted sensitivity, especially for AT-rich targets as well as difficulties with single nucleotide mismatches.

\* Corresponding author. Fax: +45 45661888.

E-mail address: [ina@exiqon.com](mailto:ina@exiqon.com) (I.K. Dahlsveen).<sup>1</sup> These authors contributed equally.

We have developed a new method for microRNA qPCR which starts with a cDNA synthesis reaction incorporating poly-adenylation and reverse transcription in a single step followed by microRNA amplification using two microRNA-specific, LNA<sup>TM</sup>-enhanced primers. The inclusion of LNA<sup>TM</sup> in the PCR primers allows the design of a short, yet highly specific forward primer covering most of the microRNA sequence. The reverse primer, also enhanced with LNA<sup>TM</sup>, covers the 3' end of the microRNA sequence. This unique combination of two microRNA-specific primers means that the system retains specificity at the same time as being extremely sensitive due to the combination of Universal RT and LNA<sup>TM</sup>-enhanced PCR.

Due to the extreme sensitivity and robustness, the new system for microRNA qPCR is especially useful for quantification of microRNAs in challenging samples. Here, we report how LNA<sup>TM</sup>-enhanced microRNA qPCR can be used to reliably detect microRNAs in difficult samples. Ready-to-use PCR panels of 730 human microRNAs enables comprehensive screening for biomarkers in substantially lower amounts of FFPE derived sample than can be achieved using microarray analysis. In addition, we report how microRNA detection can be improved by addition of a small RNA carrier prior to total RNA extraction.

## 2. Experimental methods

### 2.1. Preparation of total RNA

Total RNA from plasma was purified using the Qiagen miRNAeasy mini kit with minor modifications according to Wang et al. [9]. Carrier RNA (MS2 RNA, Roche) was added at 1 µg per 750 µl Qiazol reagent (before mixing with plasma samples). Mock purifications without plasma (but including MS2) were also performed.

Total RNA from FFPE sections including laser capture microdissected (LCM) samples were purified using the Qiagen miRNAeasy FFPE kit (50) (PN 217404, Qiagen) according to the manufacturer's instructions. One microgram of MS2 RNA (Roche) was added to each sample when indicated. A blank purification (with MS2) was included in every batch.

### 2.2. Microarray analysis

Microarray analysis was performed using miRCURY LNA<sup>TM</sup> microRNA Arrays according to the manufacturer's instructions (Exiqon). Duplicate microarrays were run using 500 ng total RNA from tumor and normal samples labeled with Hy3, against a universal reference labeled with Hy5. Signals were normalized using global Lowess and results were converted to relative values (log<sub>2</sub> ratios) between tumor and normal sample.

### 2.3. microRNA qPCR

Real-time PCR for microRNAs was performed using the miRCURY LNA<sup>TM</sup> Universal RT microRNA PCR system (Exiqon) according to the manufacturer's instructions with minor modifications (see below). For biomarker screening, Ready-to-use microRNA PCR Human Panel I and II were used (Product number 203602) according to the instruction manual (Exiqon). For each RT reaction 23 ng total RNA was used. Three RT replicates per sample were used for real-time amplification on ready-to-use PCR plates run on a Roche LightCycler<sup>®</sup> 480 real-time PCR system. Average Cq values were normalized to three stably expressed reference genes using the Exiqon GenEx software. Results were converted to relative values (log<sub>2</sub> ratios) between tumor and normal sample.

For individual microRNA assays, primer sets with the following product numbers were used: 204099, 204326, 204063, and 204230

(Exiqon). For qPCR on total RNA from plasma, 2 µl of eluted RNA (equivalent to 8 µl original plasma sample) was used in a 10 µl RT reaction. The resulting cDNA was diluted 50× and 5 µl used in 10 µl PCR amplification reactions run on a Roche LightCycler 480 real-time PCR system. For qPCR on total RNA from LCM samples, 2 µl of the 30 µl purification was used in 10 µl RT reactions as described above. Negative controls included mock purifications and no template controls.

### 2.4. Data analysis

Comparison between microarray and qPCR data was performed as described by the MAQC Consortium [12].

## 3. Results and discussion

### 3.1. microRNA biomarker screening in FFPE samples using qPCR compared to microarray

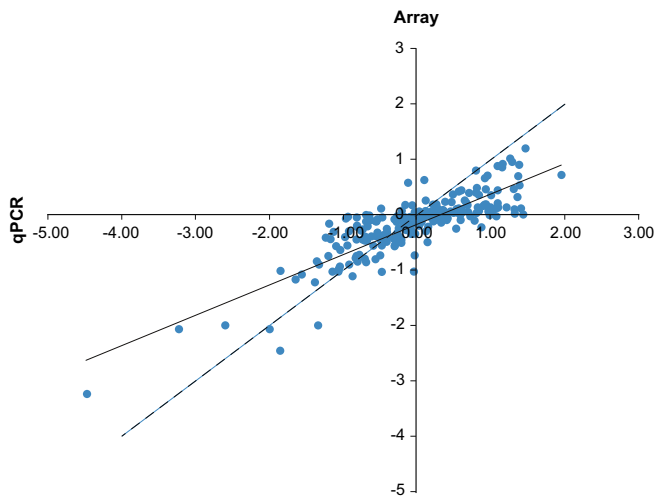
The first step in biomarker discovery is to screen for differentially expressed microRNAs in normal and disease tissue (or tissue from different stages of disease). When sufficient sample is available, initial screening using microarrays provides a comprehensive, fast and affordable method for biomarker discovery. However, microarrays are limited to a larger minimum sample amount compared to more sensitive techniques such as real-time PCR. We have compared the use of miRCURY LNA<sup>TM</sup> microRNA Arrays and LNA<sup>TM</sup>-enhanced qPCR for detection of differences between tumor and normal tissue samples.

For expression analysis using microarrays, 500 ng total RNA from normal and tumor FFPE tissue was used to screen a total of about 1300 human microRNAs. On the arrays, 16 microRNAs were shown to be more than 2-fold differentially expressed between tumor and normal samples. LNA enhanced microRNA qPCR enables similar expression analysis of 730 human microRNAs in two ready-to-use PCR plates from only 23 ng total RNA from normal and tumor FFPE tissue. Out of the 730 microRNAs, 68 were shown to be more than 2-fold differentially expressed between tumor and normal samples.

A comparison of 220 microRNAs with robust detection (signals below 36 Cq values) in qPCR and corresponding probes on the microarray shows a very good correlation between the two platforms (Fig. 1). Compared to a perfect fit between array and qPCR data, the correlation shows that qPCR enables larger differences to be detected due to a larger dynamic range. The results show that the new LNA<sup>TM</sup>-enhanced microRNA qPCR method allows biomarker screening in clinical samples using 20× less starting material and providing more extensive results compared to microarray screening.

### 3.2. Improved quantification of microRNAs in plasma and LCM sections

The small size of microRNAs makes reproducible recovery from very small amounts of challenging samples difficult. In order to improve microRNA recovery from clinical samples, we implemented the addition of a small carrier RNA prior to total RNA extraction. To test the effects of the carrier in blood plasma we quantified three microRNAs known to be expressed at different levels, hsa-miR-192, hsa-let-7a and hsa-miR-103. The addition of carrier means that the RNA in the samples cannot be accurately quantified, however, the same amount of total RNA equivalent to 8 µl of plasma was used in each RT reaction. This was considered a reasonable approach as all samples were handled in the same way from sampling of blood to further processing and RNA preparation. The improvement in microRNA quantification after addition of

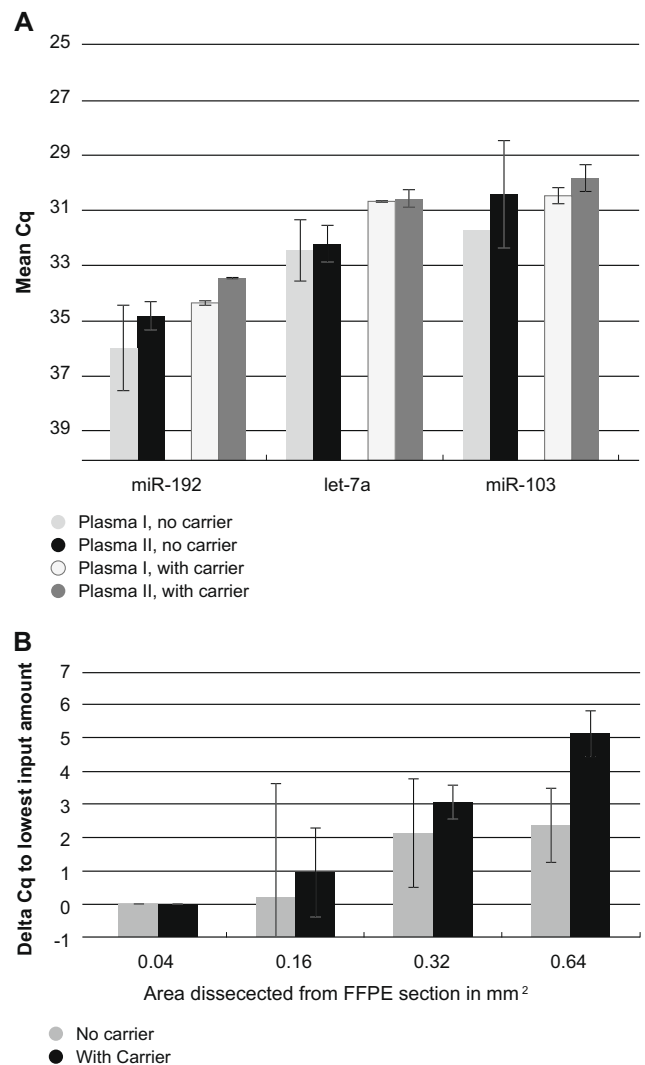


**Fig. 1.** Correlation between microRNA qPCR and microarray expression analysis. The same total RNA extracted from FFPE sections from tumor and normal samples was analyzed using either miRCURY LNA arrays or LNA microRNA qPCR. The amount of total RNA used in the microarray analysis was 500 ng per sample whilst the amount of total RNA used for qPCR was 23 ng per sample. A total of 220 microRNAs detected by qPCR and with probes available on the array are included. Log<sub>2</sub> ratios from both arrays and qPCR between tumor and normal samples were compared according to reference [12] and gives a Pearson correlation of 0.84. The linear regression (black line) compared to a perfect fit (stippled line) of the two data sets indicates that qPCR can be used to detect larger differences between samples.

RNA carrier to plasma samples is shown by an average decrease of 1–2 Cq values compared to total RNA isolated without carrier (Fig. 2A). The positive effect of the carrier was slightly higher for microRNAs with lower expression levels. In addition to lower Cq values, the addition of carrier also improved reproducibility between separate RNA extractions from an average standard deviation of 1.18 Cq values between different extractions without carrier to an average standard deviation of 0.21 Cq values between different extractions with carrier RNA. The improvement in microRNA detection is likely due to improved microRNA recovery in the presence of carrier, but additional beneficial effects of the presence of carrier cannot be ruled out. A mock extraction using the same amount of carrier RNA did not generate any signal for any of the three microRNAs tested (data not shown).

The effects of addition of carrier during RNA extraction was also tested on LCM sections from FFPE samples. Areas ranging from 0.04 mm<sup>2</sup> to 0.64 mm<sup>2</sup> (equivalent to approximately 25–400 cells) were microdissected from two FFPE sections, and in each case, two independent LCM dissections were made. Total RNA was isolated with and without carrier RNA. Three microRNAs (hsa-miR-21, hsa-miR-103, has-let-7a) were quantified using LNA<sup>TM</sup>-enhanced qPCR in each sample. In order to investigate the correlation between the area dissected and microRNA detection, the Cq values obtained from the various areas were compared to the Cq values from the lowest amount of input material (0.04 mm<sup>2</sup>). The average delta Cqs with and without the addition of carrier RNA are shown in Fig. 2B. The results show that the addition of carrier RNA prior to RNA extraction improves microRNA quantification from very small amounts of LCM starting material. The variation in microRNA quantification between separate RNA isolations is large in the absence of carrier. The addition of carrier leads to reduced variation between separate extractions. A mock isolation using the equivalent amounts of carrier, but no sample gave no signal for any of the microRNAs tested (data not shown).

The new microRNA qPCR method using Universal RT and LNA<sup>TM</sup>-enhanced primers allows microRNA quantification in



**Fig. 2.** The addition of carrier improves microRNA quantification. Quantification of three microRNAs in plasma shows that the use of an RNA carrier improves both mean Cq values and reproducibility of parallel extractions from the same sample (A). For each microRNA, two separate RNA isolations were performed from plasma I and plasma II. Error bars represent the standard deviation between parallel RNA isolations from the same sample. microRNA recovery and quantification from FFPE samples is also improved by the addition of carrier (B). Areas of varying size were isolated from FFPE sections using LCM. Duplicate RNA isolations were performed on duplicate LCM samples and three microRNAs quantified by qPCR (hsa-miR-21, hsa-miR-103, has-let-7a). The average delta Cq values compared to the lowest input amount is shown. Error bars represent variation between duplicate isolations. The inclusion of carrier RNA leads to improved recovery and quantification of microRNA as well as reduced variation.

minute amounts of challenging clinical samples such as blood plasma and LCM sections from FFPE material. Our results suggest that the addition of an RNA carrier prior to total RNA extraction improves the quantification of microRNAs in challenging samples. Inclusion of carrier probably improves recovery of microRNAs from the samples, but might also have additional beneficial effects on microRNA quantification, such as improved stability or availability in extracted RNA. The fact that the addition of carrier also seems to improve reproducibility between separate extractions is particularly interesting when considering biomarker screening and validation in large numbers of samples collected and isolated at different time points. Furthermore, the low yield of RNA in such samples may prevent RNA quantification in which case robustness in the extraction method is prerequisite.

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