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Optimizing miR-29 measurements in biobanked, heparinized samples

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ABSTRACT

Aims: MicroRNAs (miRs) and their importance in development, normal physiology, and disease have become increasingly recognized. Our laboratory is interested in miR-29 and its effects on lung development. These studies set out to identify optimal conditions for the measurement of miR-29 in heparinized, biobanked samples and to compare isoform expression patterns.

Materials and methods: The efficiency of three distinct heparinases were tested using reverse transcriptase polymerase chain reaction (RT-PCR): recombinant *F. Heparinum* heparinase I; recombinant *P. heparinus* heparinase II; recombinant *P. heparinus* heparinase III; and heparinase I (B. efferthii-derived). The effects of freeze/thaws, and the relative expression of different miR-29 isoforms were also assessed using RT-PCR.

Key findings: Our investigations determined that heparinase 1 (recombinant *F. Heparinum*) and 2 (recombinant *P. heparinus*) at 1 or 2 h incubation efficiently neutralized heparin activity and prevented interference with the PCR. Also, a single freeze/thaw did not affect the measurement of miR-29-3p but multiple freeze/thaw cycles decreased the measureable miR levels. Finally, the -3p strand was most abundantly expressed in all three isoforms in both human and mouse plasma.

Significance: Our findings illustrate that specific conditions need to be optimized for the particular miR and the type of sample being tested.

1. Introduction

Since the discovery of microRNAs (miRs), their importance in development, normal physiology, and disease has become increasingly recognized [1,2]. Currently, thousands of miRs have been identified in humans with more constantly being recognized. MiRs can be found extracellularly in both plasma (serum) and urine often encapsulated within microvesicles or exosomes [3–5]. These properties make them attractive targets for biomarkers for early disease detection. However, their small size and ubiquitous nature also makes them difficult to accurately measure and current techniques can be fraught with inconsistencies. Several factors can contribute to the accuracy of miR measurements and include sample preparation, storage, correct normalization for extraction, and polymerase efficiency, but most significant are hemolysis and contamination of plasma or serum with blood cells [6–11] or collection of samples with heparin as anticoagulant. While many more sophisticated analyses have been developed [12], isolation of RNA and quantification by reverse transcriptase polymerase chain reaction (RT-PCR) is still considered the gold standard for non-analytical chemistry based approaches that can be performed in the average laboratory [13,14]. However, heparin has been shown to interfere with polymerase activity and inhibit PCR-based measurements.

Our laboratory has identified several miRs that are suppressed in infants born preterm and has begun to identify unique techniques for restoring the expression of these miRs [15,16]. MiR-29 is one of the miRs of interest and will be used as a model for miR analyses in this report. MiR-29 has three isoforms, a, b, and c and is expressed on 2 different chromosomes. MiR-29a and b1 are found on chromosome 7 (6 in mice) and miR-29b2 and c are found on chromosome 1. Each of these miRs has two distinct strands, -5p and -3p, which designates a sense and anti-sense configuration (Fig. 1). One or both of these strands can have biological properties. MiR-29a, miR-29c, and miR-29b-3p sequences are the same for mouse and human however, miR-29b1-5p and b2-5p differ between species. To fully assess the expression of miR-29 isoforms and to optimize measurement of their expression profiles we sought to measure levels of seven individual isoforms of miR-29 in human and mouse plasma under several typical biobank conditions.

Suppression of miR-29b-3p in plasma samples from preterm infants as well as our mouse model of severe newborn chronic lung disease has

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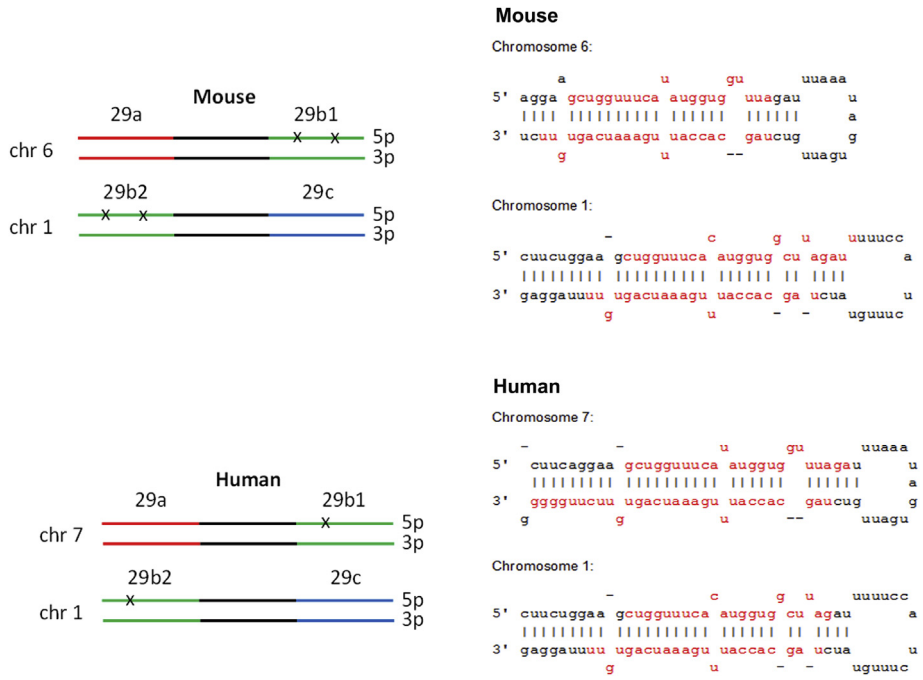

Fig. 1. Sequences for miR29 isoforms. MiR-29 a, c, and miR-29b 3p sequences are the same for mouse and human. MiR-29b1-5p and b2-5p differ between chromosomes and species. X's indicate nucleotide differences.

been previously reported [15,17]. Attempts to restore this expression in mice have resulted in normalization and restoration of the lung matrix [15]. To identify infants that would most benefit from miR-restoration therapy, we need to interrogate a large cohort of banked samples many of which are collected in heparin or have been frozen for long periods of time. This requires optimizing methods to effectively quantify the miR-isoforms in heparinized, biobanked samples.

2. Methods

2.1. Samples

Human samples were obtained from healthy volunteers using both EDTA and heparin as anticoagulants (IRB protocol #05-00338). Mouse samples were obtained from C57Bl/6 mice under light isoflurane anesthesia (IACUC protocol #AR07-00028).

Cord blood was obtained from deidentified biobanked samples obtained from preterm infants born less than 32 weeks gestation. Samples were collected and stored under IRB approved protocols (IRB #05-00338). All cord blood samples were collected in heparinized tubes and stored at -80 °C.

2.2. Heparinase

Three types of heparinases and two individual manufacturers were tested; recombinant *F. Heparinum* heparinase I (*E. coli* derived, #7897-GH-010, R&D systems Minneapolis, MN, USA); recombinant *P. heparinus* heparinase II (*E. coli* derived, #6336-GH-1010, R&D systems Minneapolis, MN, USA); recombinant *P. heparinus* heparinase III (*E. coli* derived, #6145-GH-1010, R&D systems Minneapolis, MN, USA) and heparinase I (B. efferthii-derived, #P0735S, New England BioLabs, Ipswich, MA, USA). Human samples were collected in heparin tubes (6.0 mL BD Vacutainer lithium heparin tubes containing 95 USP units), centrifuged, and the plasma divided into nine aliquots and treated with the above heparinases. After 1 h of treatment, 10 µl of sample were removed and stored at -80 °C. At 2hs the remaining sample was also stored at -80 °C until analysis.

2.3. Fresh vs frozen

Plasma samples from both mice and humans were collected and either processed fresh, after being frozen at -80 °C for 72 h, or after 5 or 10 cycles of thawing and refreezing.

2.4. Hemoglobin (Hb)

Levels of plasma free Hb were measured in plasma samples to determine the degree of hemolysis in samples using an ELISA protocol (Bethyl Laboratories, Montgomery, TX, USA).

2.5. PCR

Standard protocols for reverse transcription (RT) and qPCR were followed using miRCURY LNA Universal RT microRNA PCR Universal cDNA Synthesis kit II (Exiqon, Denmark) and miRCURY LNA Universal RT microRNA PCR ExiLENT SYBR Green master mix (Exiqon, Demark). Primer sets for sp2, hsa miR-29a-3p, hsa miR-29a-5p, hsa miR-29b-3p, hsa miR-29b1-5p, mmu miR-29b1-5p, hsa miR-29b2-5p, mmu miR-29b2-5p, hsa miR-29c-3p, hsa miR-29c-5p were purchased from Exiqon (miRCURY LNA Universal RT microRNA PCR LNA PCR primers set, Denmark) (Table 1). Where sequence homology was preserved between species, primers for human were used (Fig. 1).

Table 1
Primer sequences for PCR.

Primer	Target Sequence (5' – 3')
hsa-miR-21-5p	UAGCUUAUCAGACUGAUGUUGA
hsa-miR-29a-3p	UAGCACCAUCUGAAAUCGGUUA
hsa-miR-29a-5p	ACUGAUUUCUUUUGGUGUUCAG
hsa-miR-29b-3p	UAGCACCAUUUGAAAUCAGUGUU
hsa-miR-29b-1-5p	GCUGGUUUCAUAUGGUGGUUUAGA
hsa-miR-29b-2-5p	CUGGUUUCACAUGGUGGCUUAG
mmu-miR-29b-1-5p	GCUGGUUUCAUAUGGUGGUUUA
mmu-miR-29b-2-5p	CUGGUUUCACAUGGUGGCUUAGAUU
hsa-miR-29c-3p	UAGCACCAUUUGAAAUCGGUUA
hsa-miR-29c-5p	UGACCGAUUUCUCCUGGUGUUC

To control for differences in efficiencies at the level of cDNA synthesis and PCR, normalizers or "spike-ins" (SP6 and SP2) were added to the extraction mixture. Wells detecting RNA spike-ins were used to eliminate outlier samples and for the purpose of this study the PCR normalizer SP2 was use to compare samples.

3. Results

3.1. Heparinase treatment

Biobanked samples are often collected in heparin as an anti-coagulant and heparin is known to interfere with polymerase enzymes preventing the use of untreated samples in RT-PCR derived measurements. To identify the type(s) of heparinase that would provide optimal measurement of miRs in heparinized plasma, three types of heparinases (I, II, and III, see Methods), from 2 different companies, were tested with 2 distinct incubation times (1 and 2 h). We were not able to detect microRNA expression in plasma collected in heparinized tubes without heparinase treatment (no heparinase). Incubation with heparinase 1 or 2 yielded miR levels similar to the EDTA-treated plasma however, at 2 h, the heparinase 1 or 2 treated samples were within 1 CT of the EDTA treated sample (Fig. 2). Heparinase 3 was not effective in eliminating the detrimental effects of heparin on the plasma miR measurements.

3.2. Fresh vs frozen

To examine the effects of freezing or multiple cycles of freeze-thaws we measured miR-29b-3p in human plasma that was freshly drawn, frozen, and samples that had been through 5 or 10 cycles of freeze-thaws. Each sample was compared to the respective fresh plasma measurement. The results indicate a minimal effect of a single freeze thaw on the measurement of miR-29b-3p (Fig. 3). The effects of multiple freeze-thaws were evident and the measurements were lower after 5 or 10 cycles.

3.3. Isoform expression

To determine the relative expression of each of the miR29 isoforms we measured each in both mouse and human plasma samples. The -3p strand was the most highly expressed in all three isoforms and in both species indicating that the -3p stand is selectively stabilized while the -5p is likely degraded relatively rapidly (Fig. 4). To control for the degree of hemolysis that might be present in each of the samples hemoglobin levels were measured. Hb levels for two of the mouse samples were below the detection threshold (Fig. 5A) and the Hb levels for the human samples and the one mouse sample demonstrated no correlation to levels of microRNAs in plasma (Fig. 5B).

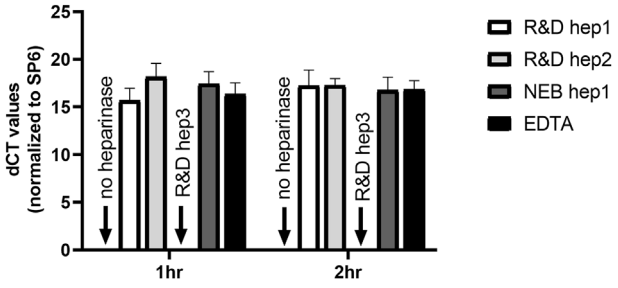

Fig. 2. Optimizing heparinases. Heparinase 1, 2, and 3 cleave heparin sulfate at different sites, thus the efficiency of each was evaluated in human samples collected in heparin. Samples were treated for 1 or 2 h with each heparinase and subsequently evaluated by RT-PCR. Data represent mean ± SD, n = 3 individual samples per treatment.

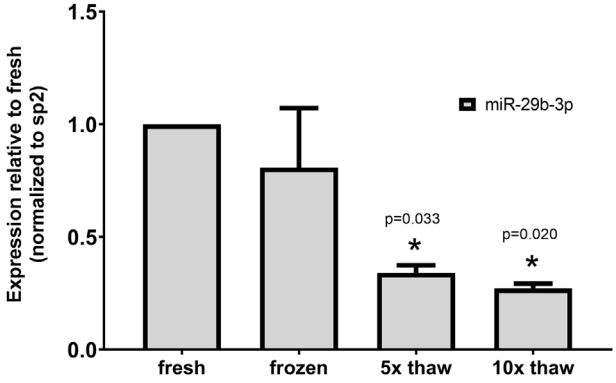

Fig. 3. Effects of freezing. The effects of freezing and multiple cycles of freeze-thaw were evaluated in human plasma by comparing each individual sample to the respective fresh measurement. There were no effects of a single freeze-thaw on the measurement of miR-29b-3p however multiple freeze-thaws did decreased detectable levels. Data were analyzed by One-way ANOVA ($p = 0.012$) with Tukey's multiple comparisons test post-hoc, $p < 0.05$. Data represent mean ± SD, n = 3 individual samples per treatment.

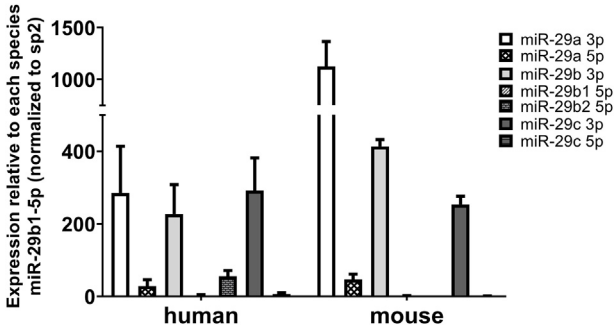

Fig. 4. Relative expression of miR-29 isoforms. The relative expression of miR-29 isoforms was evaluated in both human and mouse samples. All samples were normalized to the average dCT of the lowest expressing isoform detectable in both species, miR-29b1-5p. Data represent mean ± SD, n = 3 for each species.

3.4. Cohort data

Human cord blood samples, collected in heparin, were analyzed to identify the efficiency of our technique for biobanked samples. Samples were treated with *F. Heparimum* heparinase I for 2 h prior to RNA extraction. After RT-PCR, the pattern of expression was similar to that found in fresh frozen human plasma collected in EDTA, with greatest expression in the 3p strand of miR-29a, b, and c (Fig. 6). These data demonstrate that optimizing heparinases prior to PCR measurements will provide a viable technique for measuring biobanked samples.

4. Discussion

MicroRNAs are increasingly studied in disease and are the focus of several therapeutic strategies. However, designing interventions and assessing efficacy require accurate measurement of these miRs in biological samples. Our laboratory has been interested in the consequences of the maternal environment on preterm birth and the long-term health of the infant. We have found several microRNAs that are suppressed in infants born preterm that develop chronic lung disease and are working on therapies to restore these deficits. We are actively studying miR-29 in both humans and mice. To identify the infants most likely to benefit from miR therapy, our laboratory needed to accurately measure miRs in human biobanked samples.

Often human samples are obtained as part of a biobanked repository, are frozen for periods of time, and are collected in various anti-coagulants, the most problematic being heparin. Heparin interferes

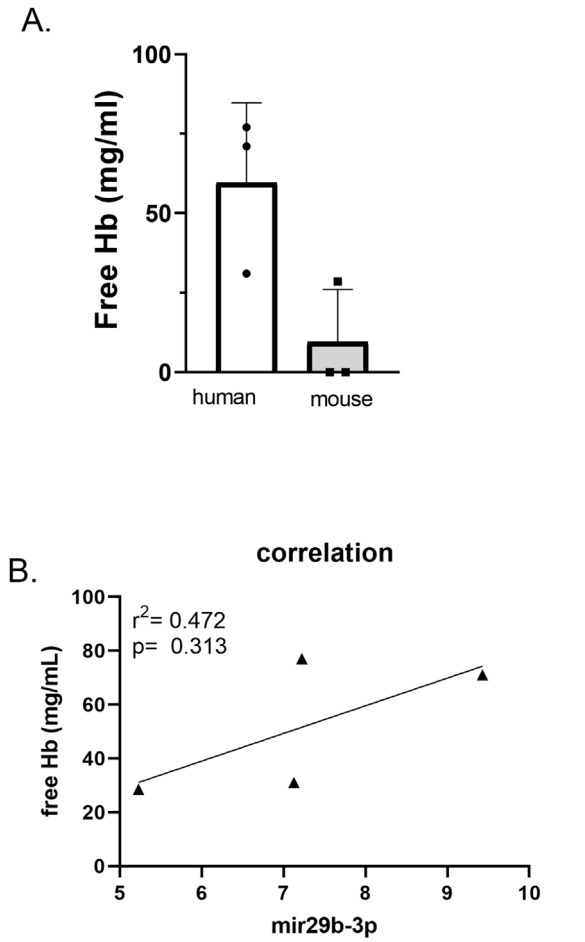

Fig. 5. Effects of hemolysis using hemoglobin measurements. Free hemoglobin was measured in both human and mouse samples. Levels were detected in all human but only one mouse sample. Omitting the 2 mouse samples that had no detectable hemoglobin, the hemoglobin levels were compared to the miR29b-3p CT (normalized to SP2) values using a Pearson Correlation. No statistical correlation was observed.

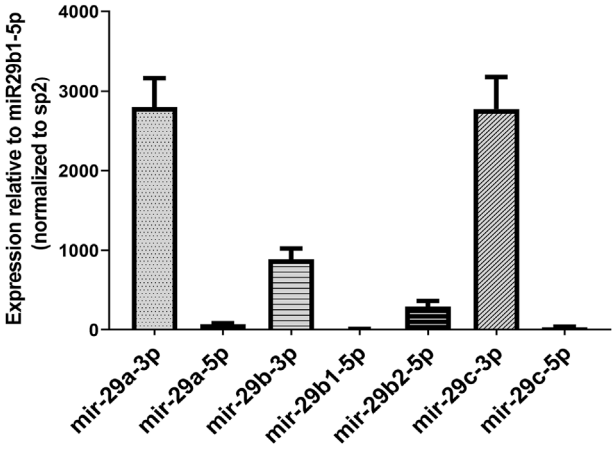

Fig. 6. MiR-29 expression in biobanked cord blood collected in heparin. The miR-29 isoforms were evaluated in cord blood samples collected in heparinized tubes. The samples were treated with heparinase I prior to RT-PCR measurements. The relative expression levels of miR-29 isoforms were similar to that observed in freshly frozen human samples. Data represent mean ± SD, n = 10.

with DNA amplification by inhibiting polymerase activity. Several reports have detailed techniques, primarily using heparinases, to attenuate this interference and allow accurate measurement of miRs. We chose to investigate the effects of 3 different available heparinases on their ability to neutralize heparin activity. Our investigations found that heparinase 1 (recombinant *F. Heparinum* heparinase) and 2 (recombinant *P. heparinus* heparinase) at 1 or 2 h incubation efficiently neutralized heparin activity sufficiently to prevent interference with the PCR (Fig. 2). We did observe that heparinase 3 (*P. heparinus* heparinase) was ineffective.

Biobanked samples generally have been frozen for periods of time and may have been subjected to freeze/thaw cycles. Our study suggests that a single freeze/thaw does not affect the measurement of miR-29-3p but that multiple freeze/thaw cycles decreases the measureable miR levels (Fig. 3). Consequently, measurement of miR-29 in biobanked samples are accurate if frozen but should not be subjected to thawing.

Mir-29 has 3 isoforms found on 2 chromosomes. To identify the relative expression of the miR-29 family, we measured levels of both strands -3p and -5p of miR-29a, b, and c in both mice and humans. As noted in Fig. 4, the -3p strand is most abundantly expressed in all three isoforms. These differences are even more exaggerated in mice as the only -5p strand at a measureable level was 29a-5p. These data imply that the -3p strands are selectively stabilized possibly by interactions with the RNA-induced silencing complex (RISC) and are likely to play a greater role in transcriptional regulation [18]. Detectable levels of Hb were measured in 3 human and 1 mouse sample (Fig. 5A). However, in our studies (with small numbers) there was no correlation between Hb levels and miR levels (Fig. 5B).

Finally, we tested our techniques on a cohort of biobanked cord blood plasma samples collected in heparin and stored for greater than 5 years. Our results indicate the all isoforms and strands of miR-29 are measurable and the levels exhibit the same pattern of relative expression as observed with the freshly frozen plasma (Fig. 6). Interestingly, the absolute expression of the biobanked samples collected from cord blood of preterm infants was substantially greater than the fresh samples collected from healthy adults but the significance of these differences is not known.

Limitations to our study include that the findings may not be applicable to all miRs. Decreased stability and degradation are likely to influence the measurement of other miRs in a differential manner. We also did not independently test primer efficiency and some differences in relative expression between miR isoforms may be due the specific primers used. Overall, our findings illustrate that measuring miRs can be fraught with many variables and that the specific conditions need to be optimized for a particular miR and the type of sample being tested.

Author contributions

Conceptualization CMW, MJC, LKR; Analyses CMW, MJC; wrote original Draft, CMW; review and editing MJC, LKR.

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