Bachelor Degree Project

UNIVERSITY OF SKÖVDE

METHOD DEVELOPMENT FOR SEPSIS BIOMARKERS

Detection and quantification of extracted microRNA using the novel Two-tailed RT-qPCR method

Bachelor Degree Project in Bioscience
G2E level, 30 ECTS
Spring term 2020

Isabelle Reihell
a15isare@student.his

Supervisors:
Anna-Karin Pernestig
anna-karin.pernestig@his.se
Sanja Jurcevic
sanja.jurcevic@his.se

Examiner:
Magnus Fagerlind
magnus.fagerlind@his.se

School of Bioscience
University of Skövde
Box 408
541 28 Skövde

2020-01-20 – 2020-06-07
Version 2

Abstract

Sepsis is a condition of the host's extreme response to an infection affecting multiple physical functions which can lead to septic shock and death. Currently, the method for diagnosing sepsis is time-consuming and relies on multiple non-specific biomarkers such as Procalcitonin, lactate and C-reactive protein, with blood culturing being the golden standard. Recently proposed and possible sepsis-specific biomarkers are the microRNAs, which are short, ~22 nucleotide long non-coding RNAs that regulate gene expression post transcriptionally. MicroRNA expression levels have the advantage of being more specific to certain disease types, making them promising biomarkers for sepsis diagnostics. The aim of this study was to evaluate the method and performance of microRNA extraction, using microRNA spike-ins to increase concentration of said extractions, and microRNA amplification by using the novel two-tailed RT-qPCR method developed by TATAA Biocenter. Blood gathered from self-assessed healthy donors was centrifuged to collect plasma, from which microRNA extraction was performed using the miRNeasy Serum/Plasma Advanced kit (Qiagen). Each sample was spiked with the synthetic microRNAs miR-223 or miR-let7b either before or after RNA extraction. The two-tailed RT-qPCR method was used to amplify the spiked-in microRNAs miR-223 and miR-let7b present in the extractions, and absolute quantification was used to determine the amount of those microRNA. The results conclude that spiked-in microRNA from plasma could be amplified using the two-tailed RT-qPCR, and the results depended on which synthetic microRNA had been added, at what concentration and at what stage of the extraction process.

List of abbreviations

BP	Base pair
CRP	C-reactive protein
Cq	Quantification cycle
cDNA	Complementary DNA
dsDNA	Double stranded DNA
dsRNA	Double stranded RNA
K2EDTA	Dipotassium Ethylenediaminetetraacetate
mRNA	Messenger RNA
miRNA	MicroRNA
NLCR	Neutrophil-lymphocyte count ratio protein
No-RT	No reverse transcriptase
NTC	Non-template control
NT	Nucleotide
PCT	Procalcitonin
qPCR	Quantitative Polymerase Chain Reaction
RT	Reverse transcription

Table of contents

Introduction

Sepsis, previously more known as blood poisoning, is a life-threatening condition which arises due to the body's dysregulated response to infections. These infections are caused by bacteria, fungi, viruses and parasites but most commonly bacteria, such as *Escherichia coli*, *Klebsiella pneumoniae* and *Staphylococcus aureus* (Karthikeyan & Premkumar, 2001). However, the current diagnostic method of identifying sepsis before treatment is a challenge. The diagnostic method depends on a few different biomarkers. Biomarkers are measurable indicators of severity or presence of disease state. Biomarkers can measure physiological components such as blood pressure and body temperature, and molecular components such as antibodies in serum and plasma, to name a few. Currently, the most common biomarkers for diagnosing sepsis are the measuring of C-reactive protein (CRP), procalcitonin (PCT) and culturing organisms in blood in bottles containing growth media for anaerobic or aerobic organisms, which is seen as the current gold standard. These methods often come short since they either do not distinguish between bacterial sepsis and other inflammatory symptoms, or the process is very time consuming. The accuracy of measuring CRP and PCT have been questioned because of their inconsistent results depending of the severity of the illness. Blood culturing is the preferred method since it can detect the pathogens in the blood that cause sepsis, but this process usually takes up to two days (Lambregts et al., 2019). Lactate is also used as a biomarker for sepsis but lacks in specificity (Ljungström et al., 2017). The ratio of neutrophil and lymphocyte count (NLCR) is a recent diagnostic method that shows a correlation with affliction severity (Ljungström et al., 2017). It is the combination of these different diagnostic biomarkers that give an indication about sepsis today, because yet, no single method is precise enough on its own (Ljungström et al., 2017). The unavailability of a specific diagnostic test for sepsis also contribute to the administration of broad-spectrum antibiotics, which can increase the mortality of the patients when not given the correct antibiotic or treatment and contribute to antibiotic resistance (Kingsley & Bhat, 2017).

A more recent proposed biomarker for diagnosing sepsis is microRNA (miRNA). In the regulation of gene expression post-transcriptionally, miRNAs are the ones in charge. By sequence-specific binding, miRNAs regulate by degrading or blocking translation of messenger RNAs (mRNAs) (Pogribny, 2017). MiRNAs comprise approximately 1-3% of the entire genome and are about 20-24 nucleotides (nt) in length. They are transcribed by RNA polymerase II and III as primary microRNAs, and become precursor miRNAs by cleaving and are processed into miRNA duplexes by double stranded RNA (dsRNA) and later into mature miRNA (Pogribny, 2017). There are currently over 2000 miRNAs that have been identified in humans (Alles et al., 2019) and they are detectable and highly stable in body fluids such as plasma and serum, and this stability is likely due to encapsulation of the miRNAs by vesicles such as exosomes and other protein complexes (Kirschner, Zandwijk & Reid, 2013).

The post-transcriptional expression of miRNA; their ability to hybridize target messenger RNAs (mRNA) to regulate their translation and stability (Filipowicz et al., 2008), occurs in different areas of the body, such as organismal development, homeostasis and neurobiology. Some diseases can alter levels of miRNA, thus making miRNAs as possible biomarkers for disease. These diseases include, but are not limited to, different types of cancer, cardiovascular disease, neurodegenerative disease and sepsis (Pogribny, 2017). It is the nature of these highly stable small RNAs that has made them such promising candidates for sepsis diagnostics (Pogribny, 2017).

MiRNAs have been observed displaying varying levels of expression during sepsis. MiRNAs such as miR-133a, mirR-155, miR-150 and miR-193 have shown to have an increased expression within a murine model affected with sepsis (Correia et al., 2017). MiR-133a, as well as miR-122, have shown an increased level of expression in humans in the presence of sepsis as well (Correia et al., 2017). MiR-150 in particular has been shown to have heavily dysregulated expression levels in leukocytes from plasma samples derived from septic patients, when compared to miR-192 as a reference gene that has no variation in the presence of sepsis (Vasilescu et al., 2009). The findings of Vasilescu et al also show a great dysregulated level of expression of miR-182 and miR-342-5p from leukocytes in septic patients.

Other miRNAs that have shown different activity in terms of expression are miR-297 and miR-574-5p, where the former is upregulated and the latter is downregulated, when measured in non-surviving sepsis patients (Correia et al., 2017). Together with the sequential organ failure assessment (SOFA) score, which is a method for describing organ failure in critically ill patients (Vincent et al., 1998), the sepsis stage and the expression of miR-574-5p, has been shown to be a reliable predictive tool for patient survival from sepsis (Correia et al., 2017).

There is a lot of promise with using miRNAs in the diagnosis of sepsis. They are suggested to distinguish sepsis's different stages and its different features, hopefully leading to an earlier diagnosis in which blood culturing, a time-consuming process, will not be crucial in the early moments of diagnosis (Kingsley & Bhat, 2017). Although it's promise, there is currently no consensus as to how miRNAs can be used as effective biomarkers for sepsis. Currently, common normalization strategy for miRNA is based on endogenous miRNAs and exogenous oligonucleotides (Faraldi et al., 2019). This means that a normalization as well as a standardization for data need to be brought forward. It is also important to realise that most studies done on miRNA within diagnosis are not sepsis specific (Kingsley & Bhat, 2017).

In order to quantify and assess the purity of miRNA from any blood, it is extracted and isolated from human fluids such as plasma. It can be a challenging task to extract pure miRNA, due to the various contaminating proteins present in the plasma, the low concentration present in the plasma, or even the configuration of the miRNA itself (Wright, de Silva, Purdie & Plain, 2020). Furthermore, it is of importance to consider which extraction method and commercial kit to use when performing the extraction of miRNA. Commercial kits that have been studied and compared in miRNA extraction include, but are not limited to: miRNeasy Serum/Plasma kit (Qiagen), Quick-cfRNA Serum & Plasma kit (Zymo Research), Isolate II RNA Micro Kit (Bioline) and PureLink RNA Mini Kit (Thermo Fisher Scientific) (Wright, de Silva, Purdie & Plain, 2020). There is currently no standardized method for performing miRNA extraction (Wright, de Silva, Purdie & Plain, 2020).

In order to see if specific miRNA has been successfully extracted, the reverse transcription quantitative PCR (RT-qPCR) is a common method to achieve this (Applied Biosystems, 2004). MiRNAs are usually present in very small concentrations in plasma, which requires a highly sensitive method such as the RT-qPCR. (Faraldi et al., 2019). It synthesizes complementary DNA (cDNA) that is later amplified in a PCR. The qPCR is performed to measure the quantity of cDNA. In the qPCR, quantification cycles (Cq), are used to measure gene expression and are generated by measuring targets in reference to a standard curve, which needs to be included in the qPCR plate for each reading, which also works as a positive control that is constituted by a series of dilutions of samples with known concentrations on a logarithmic scale. This method of measuring gene expression is known as absolute quantification. Another method of measuring gene expression is relative quantification, which uses fold-change or a fold-difference in relation to a reference gene, to generate expression levels (Applied Biosystems, 2004).

While the gold standard for nucleic acid detection is qPCR, another method known as isothermal amplification has risen as an alternative for miRNA detection that does not require temperature cycling and works well with short RNA and DNA (Deng, Zhang & Li, 2017). Techniques developed for this method are based on enzyme-based replication, digestion, and enzyme-free strand displacement processes. It has both linear and exponential amplification, which have been built from multiple primers that are comparable to PCR (Deng, Zhang & Li, 2017). As with most miRNA detection methods, isothermal amplification has no standardized reference gene or method. However, isothermal amplification is a reliable method that challenges the golden standard that is the qPCR (Deng, Zhang & Li, 2017).

The most common ways of measuring miRNA expression are microarrays, next generation sequencing and the RT-qPCR. Microarrays has the advantage of being able to monitor a large number of targets and is more cost efficient than next generation sequencing, but its specificity is limited (Androvic, Valihrach, Elling, Sjoback & Kubista, 2017). Next generation sequencing has its advantage in being the only method of discovering new miRNAs, but the cost is high and the method is complex. RT-qPCR is a reliable method when needing high precision, and preferable when the number of targets is low. The drawback, however, is that the miRNA molecules are the same size, 19-24 nt long, as a PCR primer (Androvic, Valihrach, Elling, Sjoback & Kubista, 2017).

The two-tailed RT-qPCR is able to capture a full accurate representation of the miRNA molecule with the help of target-specific, short RT-primers composed of probes that bind to the miRNA, and uses a second probe to ensure the sensitivity and specificity (Figure 1) (Androvic, Valihrach, Elling, Sjoback & Kubista, 2017). There are a few variations on how to quantify miRNA using RT-qPCR, which can be split into two groups based on either specific reverse transcription or universal. However, it is the two-tailed RT-qPCR that offers higher sensitivity, the ability to quantify isomiRs as well was being able to distinguish between different, yet similar miRNAs (Androvic, Valihrach, Elling, Sjoback & Kubista, 2017).

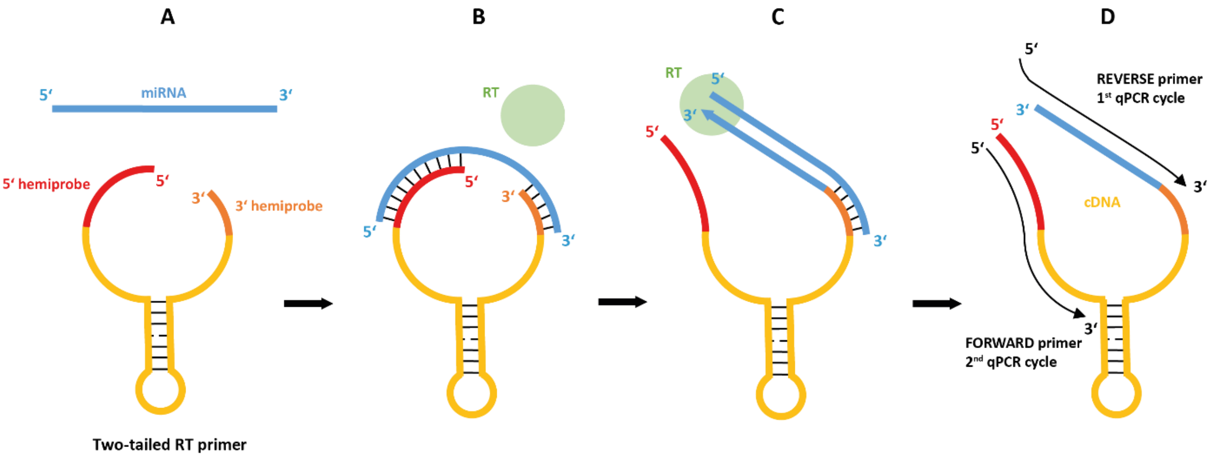


Figure 1. Copyrighted by Androvic et al. Schematic of Two-Tailed RT-qPCR. (A) The two-tailed RT primer connects two hemiprobes by a hairpin folding sequence to prevent unspecific binding, the longer 5'-hemiprobe connecting to the 5'-region of the targeted miRNA, and the 3'-hemiprobe connecting to the 3'-region. (B) The two hemiprobes bind cooperatively to each end to form a complex. (C) The cDNA is formed by reverse transcriptase binding to the 3'-end and the RT enzyme replaces the 5'-end. (D) The cDNA is finally amplified with the qPCR using the two target-specific primers.

Using the two-tailed RT-qPCR and optimizing it to be performed by hand using smaller volume such as 100 µl, and to analyse different types of miRNAs extracted from human plasma seems promising and has the feasibility to generate a better understanding of the use of miRNAs in sepsis diagnosis.

Aim and objective

The aim of this thesis project was to assess functionality and effectivity regarding miRNA extraction and miRNA quantification of extracted miRNA from plasma using the new two-tailed RT-qPCR method. The two-tailed RT-qPCR developed and studied by TATAA Biocenter has been successful in detecting miRNAs thanks to its specific two-tailed primers, which is why it is of interest to study different applications of the method. The research question for this project is if it is possible to follow and conduct an experiment following TATAA Biocenters protocol by hand, and using only 100 µl of plasma. The plasma used for the miRNA extraction samples has been spiked with either the synthetic miRNAs miR-223 and miR-let7b, to ensure that the two-tailed primers can detect quantifiable amounts of miRNA.

Thus, the project includes the collection of blood and isolation of plasma by centrifugation, the usage of the synthetic miRNA miR-223 and miR-let7b as spiked-in controls at different concentrations that were added either before or after the miRNA extraction determine the efficiency of the extraction kit and to assure detectable levels of miRNA, and finally performing a reverse transcription and quantification within the two-tailed RT-qPCR, using the absolute quantification method.

The project is a small part and continuation of the 'Future Diagnostics of Sepsis' research currently being done at the University of Skövde. This research involves discovering and evaluating promising biomarkers to create a multi-marker panel for the diagnosis of bacterial sepsis, to reduce sepsis mortality and complications. The objective for this thesis; to find out if it is possible to identify and quantify miRNA from plasma, can they be used to further the studies of the usage of miRNA as biomarkers, and evaluate their usage in diagnostics within sepsis, and lastly to determine specificity and usage of the two-tailed primers in two-tailed RT-qPCR.

Materials and methods

The procedure was divided into two different variations and are visible in Figure 2a and Figure 2b. Blood was collected from healthy blood donors in 6 ml K2E K2EDTA vacuette tubes (Greiner Bio-One) and was centrifuged for 15 minutes at 2,000 x g speed using a ScanSpeed 1580R (Labogene) in order to remove cells and deplete platelets (ThermoFisher, 2007). The plasma was collected and was aliquoted into 30 new tubes and stored at -80 °C. To extract miRNA from the plasma, plasma was thawed in a 37°C water bath (VWR) until completely thawed. The plasma was centrifuged again as per the miRNeasy Serum/Plasma advanced kit protocol recommendation for 5 minutes at 12,100 x *g*.

The starting volume of the samples were 200 µl, as recommended by the miRNeasy Serum/Plasma advanced kit (Qiagen). In the first procedure (Figure 2a), 1,8 µl plasma was removed from the unextracted samples and 1,8 µl of synthetic miRNA with different copy numbers were added as visible in Figure 3 in order to achieve a ratio of 1:10. By adding 1,8 µl of synthetic miRNA with 10^7 copy numbers per µl to the unextracted sample, a 10^6-copy number per µl concentration was achieved. Similarly, to achieve the concentration of 10^5 copy numbers per µl, the 10^6 copy numbers per µl stock solution was added. This was done to compare the spike-in efficiency from the values given by the qPCR later on. The extraction of miRNA was done according to the miRNeasy Serum/Plasma Advanced Kit (Qiagen). The final eluate had a volume of 18 µl.

In the second procedure (Figure 2b), extraction of miRNA was done according to the miRNeasy Serum/Plasma Advanced Kit (Qiagen), and 1,8 µl was removed from the eluate and 1,8 µl of the synthetic miRNA was added as visible in Figure 3. The final eluate had a volume of 18 µl.

After the miRNA had been extracted, the miRNA was quantified using Qubit microRNA assay kit (Thermo Fisher Scientific) in order to detect and measure the RNA present. The sample was prepared according to the Qubit user guide (Thermo Fisher Scientific) and then measured using a Qubit fluorometer (Thermo Fisher Scientific).

The sample's purity was also measured using the NanoDrop 2000 Spectrophotometer (Thermo Fisher Scientific) using a 260/280 mm-absorbance ratio for RNA.

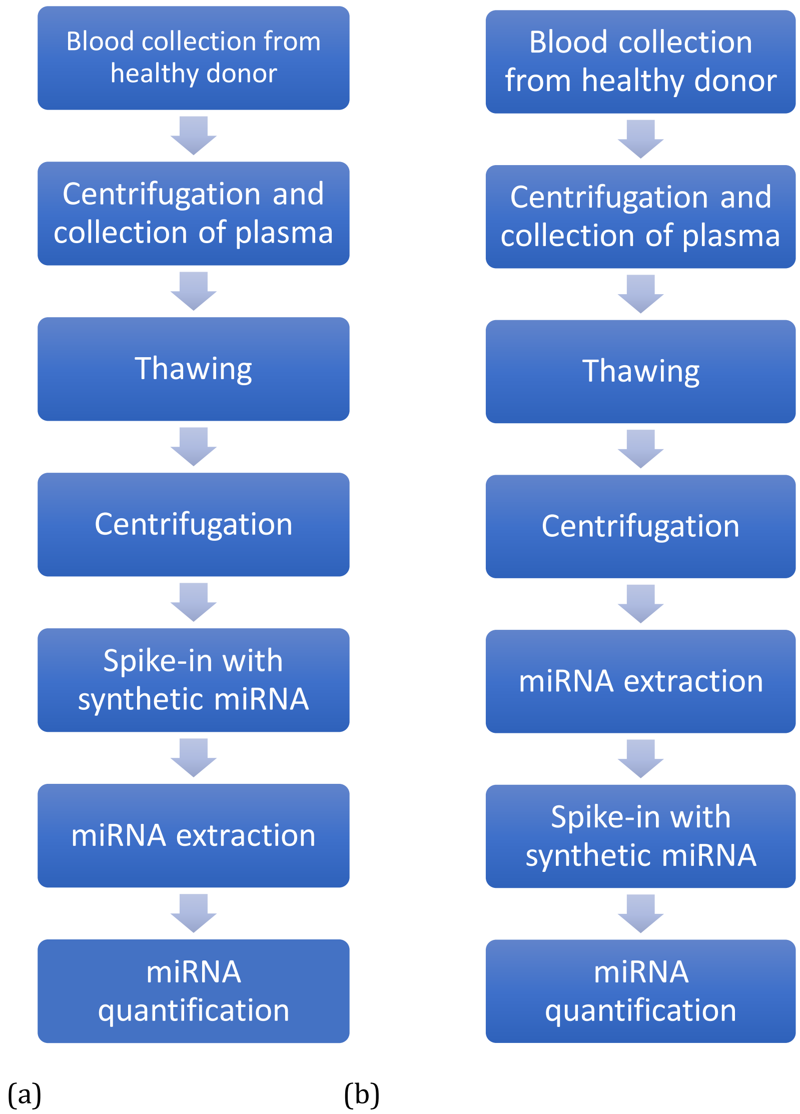


Figure 2. Workflow of the spike-in procedure before miRNA extraction (a) and workflow of the spike-in procedure after miRNA extraction (b).

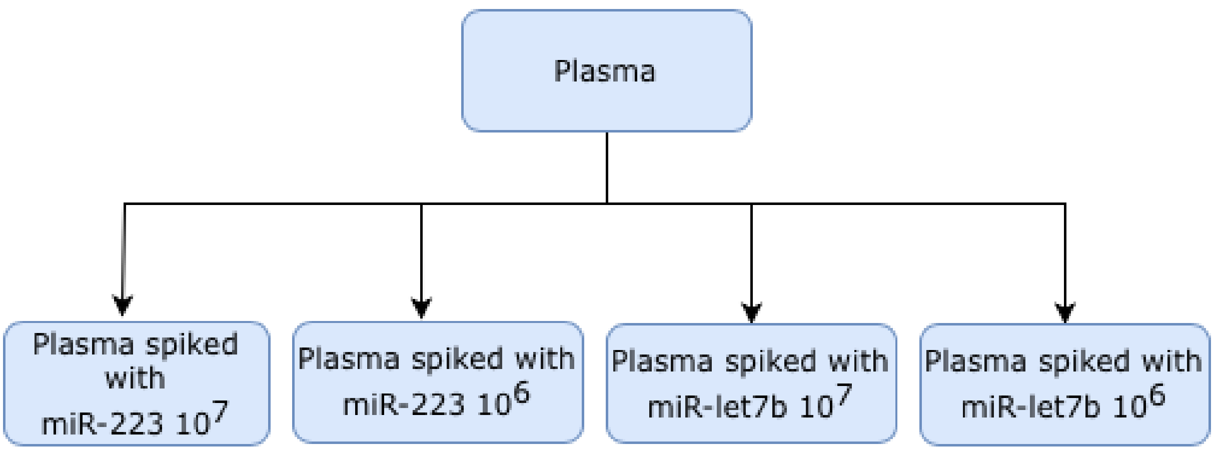


Figure 3. The different types of spike-ins using synthetic miRNA miR-223 and miR-let7b with the copy numbers of 10^6 and 10^7.

Reverse transcription

Reverse transcription (RT) was carried out according to the Two-tailed RT-qPCR protocol (TATAA Biocenter), using the TATAA GrandScript cDNA FreePrime kit (TATAA Biocenter) in order to create complementary DNA (cDNA). The RT-reactions were prepared as shown in Table 1. To achieve the 0.2 µM concentration needed for the RT primer, 0.5 µl (100 µM) was mixed together with 249.5 µl nuclease free water. In addition to the reverse transcriptase reactions, a no reverse transcriptase (no-RT) control and non-template control (NTC) was carried out as well. The no-RT control contained nuclease free water instead of the GrandScript RT Enzyme to determine if the reagents were contaminated with genomic DNA, and the NTC only contained nuclease free water instead of the RNA sample to determine if the sample was contaminated. All of the reactions were run in a PCR thermocycler according to the steps described in Table 2.

Table 1. Components used to create cDNA.

Component	Volume (µl)
GrandScript FreePrime Reaction Mix (5x)	2
GSP enchancer (10x)	1
GrandScript RT Enzyme	0.5
Two-tailed RT primer (0.2 µM)	2.5
RNA sample	4
Total	**10**

Table 2. Conditions for the PCR running the reverse transcription.

Temperature (°C)	Time (min)
42	45
85	5
4	hold

Quantitative PCR

Quantification of the template cDNA was performed according to the two-tailed RT-qPCR protocol (TATAA Biocenter) shown in Table 3, using the TATAA SYBR® GrandMaster® Mix (TATAA Biocenter). Since the TATAA master mix does not contain a reference dye needed for sample fluorescence, 0.2 µl of ROX Reference Dye (Thermo Fisher Scientific) was added to the cDNA reaction mix to get the working concentration of 500 nM, for fluorescent signaling normalization to reduce variations in fluorescent values.

Table 3. Components used for qPCR reactions.

Component	Volume (µl)
TATAA SYBR® GrandMaster® Mix (2x)	5
Two-tailed PCR forward primer (10 µM)	0.2
Two-tailed PCR reverse primer (10 µM)	0.2
cDNA	2
Nuclease free water	2.6
ROX Reference Dye (500 nM)	0.2
Total	10.2

Each reaction was prepared in triplicates. The qPCR 96-well plate (Applied Biosystems) included eluates from the spiked miRNA samples, synthetic miRNA for standard curve, no reverse transcription controls as well as non-template controls. When the samples were loaded the plate was vortexed and centrifuged at 350 x *g* for 1 minute in a 320 centrifuge (Hettich). The qPCR was run in the 7300 Real-Time PCR System (Applied Biosystems) according to the qPCR cycling program showed in Table 4.

Table 4. qPCR cycling protocol. Data collection was obtained during the last step of the second cycle.

Temperature (°C)	Time (s)	
95	30	
95	5	⍰ 40X
60	15	
72	30	-> data collection

Standard curve

To be able to quantify the amplification of miRNA in the RT-qPCR, the synthetic miRNA miR-let7b and miR-223 was diluted in tenfold dilutions using nuclease free water from 10^{12} to 10^5. All dilutions from 10^{12} to 10^5 was pipetted in triplicates.

In order to detect the amplification of the different miRNAs miR-223 and miR-let7, absolute quantification and melting curve analysis was performed.

Statistical analysis

Statistics including the mean concentration and mean purity for the different samples spiked with either miR-223 and miR-let7b shown in Table 5-8 were compared to determine which samples to use for the RT-qPCR.

Ethical considerations

Some ethical considerations need to be made since this experiment will be using plasma derived from human blood. However, the blood that was used is voluntarily given by self-assessed healthy thesis students and supervisors that either partake in the project or are involved by other means. Their personal information will be replaced with letters or other simplified identification means that do not involve their real names. Results and interpretations gained from the experiment and will not take the participating blood donors overall health into concern. The blood will be drawn by a licensed nurse at the university's department for nursing, which ensures the safety of everyone. The blood will be used for this experiment only, thus ensuring that everyone who gives blood has been acknowledged of the purpose. The results will not be presented to them either so consent is not needed. The integrity and privacy of whoever donates blood will be kept undisclosed.

Results

Quantity

In order to see presence of miRNA of the different samples, Qubit microRNA Assay kit (Qubit Technologies) was used. The mean concentration of the samples that have been spiked with miRNAs miR-223 and miR-let7b respectively and the different methods (see Figure 2 and 3) are shown in Table 5 and 6. All of the sample's concentrations are presented in Appendix 1. Pre indicates the samples that were spiked before extraction, Post indicates samples that were spiked after extraction. 10^6 and 10^5 indicate the copy numbers of the spike-ins. For miR-223, the samples that were spiked post extraction with the copy number 10^6 show the highest concentration (Figure 4), and the samples that were spiked post extraction with the copy number 10^5 had the lowest (Figure 5a). For miR-let7b, the highest concentration samples had been spiked pre-extraction with the copy number 10^6, and the lowest concentration samples were the post extracted samples with copy number 10^5 added (Figure 6a).

Table 5. Mean concentration of three samples spiked with miR-223, per method and copy number

Sample	Mean concentration of miRNA, ng/µl ± STDEV
Pre, 10^6	0,733 ± 0.122
Pre, 10^5	0,820 ± 0.197
Post, 10^6	0,840 ± 0.052
Post, 10^5	0,593 ± 0.046

Table 6. Mean concentration of three samples spiked with miRNA-let7b, per method and copy number

Sample	Mean concentration of miRNA, ng/µl ± STDEV
Pre, 10^6	0,993 ± 0.197
Pre, 10^5	0,773 ± 0.080
Post, 10^6	0,686 ± 0.041
Post, 10^5	0,653 ± 0.050

Purity

In order to measure the purity of the different samples, Nanodrop 2000 Spectrophotometer was used. The mean value of the samples' purity that have been spiked with miRNAs miR-223 and miR-let7b respectively and the different methods (see Figure 2 and 3) are shown in Table 7 and 8. All of the sample's purity are presented in Appendix 2. For both miR-223 (Figure 5b) and miR-let7b (Figure 6b), the highest purity was measured in the post spiked samples with the copy number 10^6, and the lowest purity were measured in the pre spiked samples with the copy number 10^5.

Table 7. Mean purity of three samples spiked with miRNA miR-223, per method and copy number

Sample	Mean value of miRNA purity, 260/280 ratio
Pre, 10^6	1.09
Pre, 10^5	0.74
Post, 10^6	1.23
Post, 10^5	1.22

Table 8. Mean purity of three samples spiked with miRNA miR-let7b, per method and copy number

Sample	Mean value of miRNA purity, 260/280 ratio
Pre, 10^6	1.52
Pre, 10^5	0.93
Post, 10^6	1.83
Post, 10^5	1.32

Cycle quantification and copies

When comparing the efficiency of the different spike-in methods (Figure 2a and 2b) of adding synthetic miRNA miR-223 to the sample either before or after miRNA extraction, the method of adding synthetic miRNA after extraction resulted in a higher concentration after the RT-qPCR was performed, and thus a larger yield of miRNA. This is visible in Figure 4. The melt curve analysis (data not shown) showed a single peak for samples spiked with miR-223 and multiple peaks for miR-let7b.

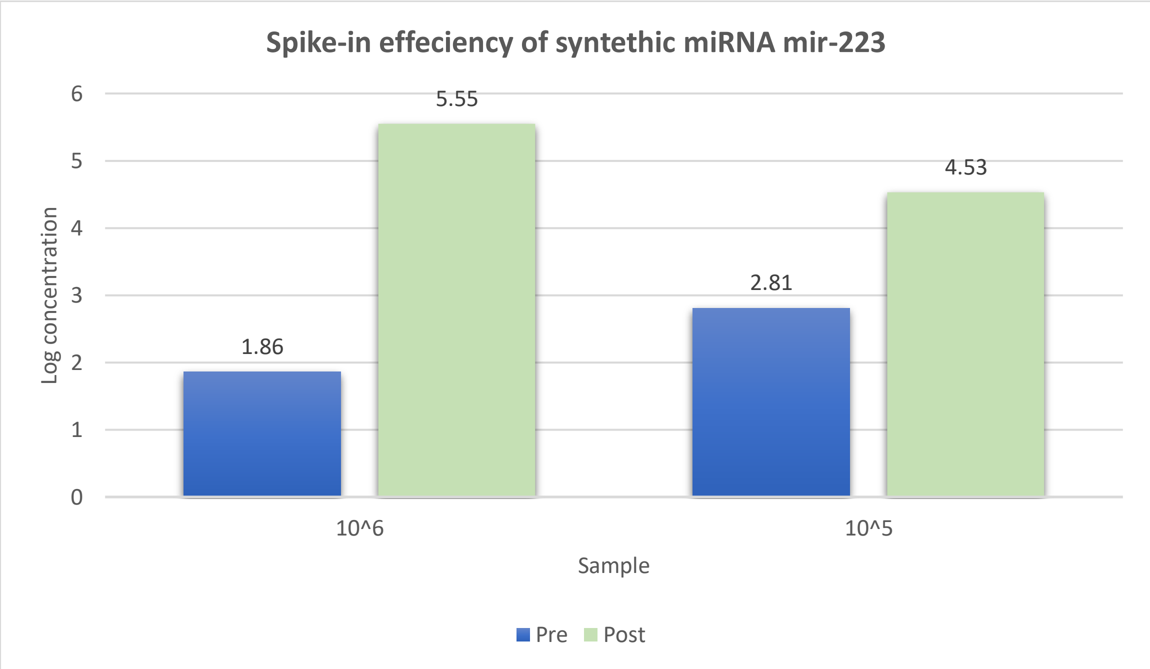
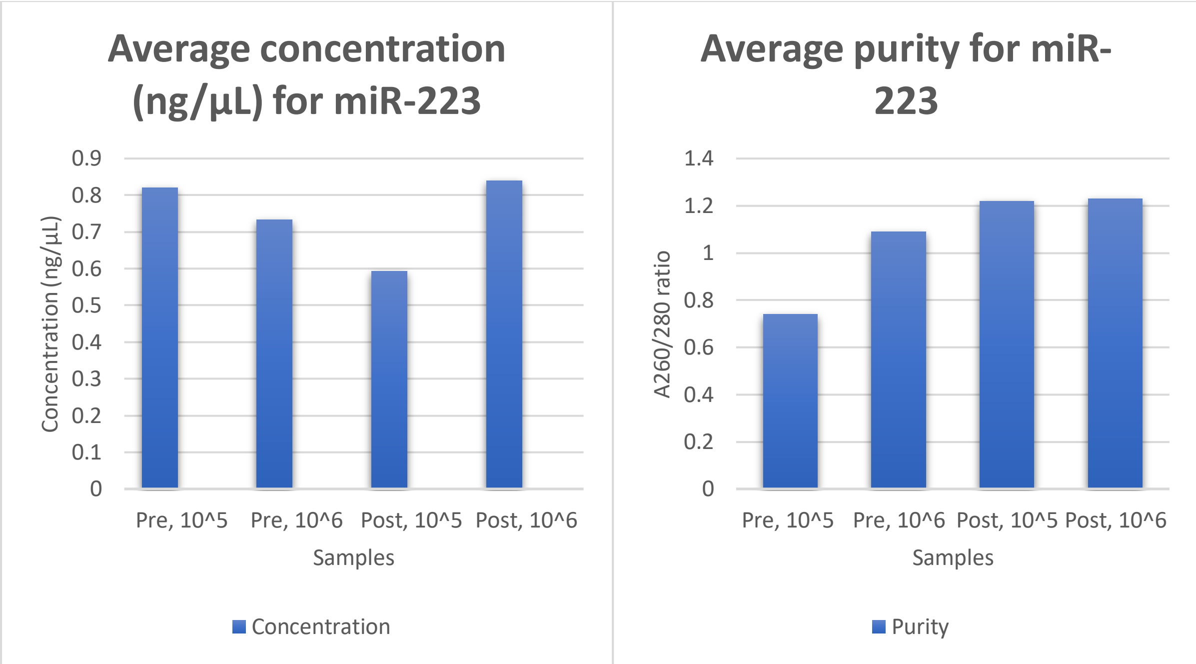
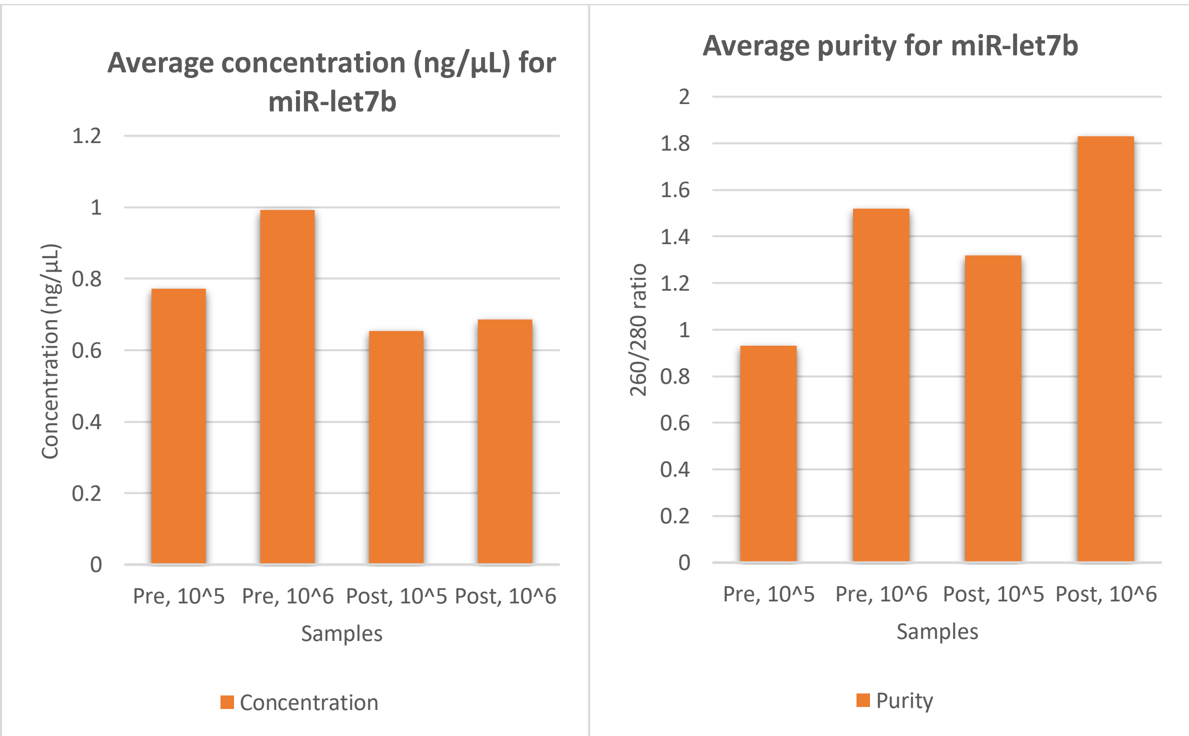


Figure 4. Graph showing the added spike-in log concentration 10^6 and 10^5, the pre-extraction log concentration and the post-extraction log concentration from the RT-qPCR.



(a) (b)

Figure 5. Graphs depicting the differences in concentration (ng/µl) (a) and purity (b) for miR-223 in post- and pre-extraction of miRNA and the added spike-ins.



(a) (b)

Figure 6. Graphs depicting the differences in concentration (ng/µl) (a) and purity (b) for miR-let7b in post- and pre-extraction of miRNA and the added spike-ins.

Absolute quantification was used to determine if the two-tailed RT-qPCR was successful in quantifying miRNA. The two different miRNA, miR-let7b and miR-223 both gave results, albeit quite different from each other.

The results from the standard curve of miR-let7b showed a linearity of 0.00018 and amplification within the non-template controls as well as in the no reverse transcriptase controls. Therefore, the test sample results of miR-let7b from the RT-qPCR remain inconclusive (data not shown).

The results from the standard curve of miR-223 showed a linearity, a R^2-value, of 0.982 (Figure 7) which evaluates the PCR efficiency. The amplification efficiency was calculated using the formula
$E = (10^{-1/slope}-1) * 100$

The slope for miR-223 was -3.3 which when calculated $(10^{-1/-3.3}-1) * 100$ gives an amplification efficiency of 100% (Figure 7).

The non-template controls and no reverse transcriptase controls showed no amplification. The results of the test samples of miR-223 are shown in Table 9.

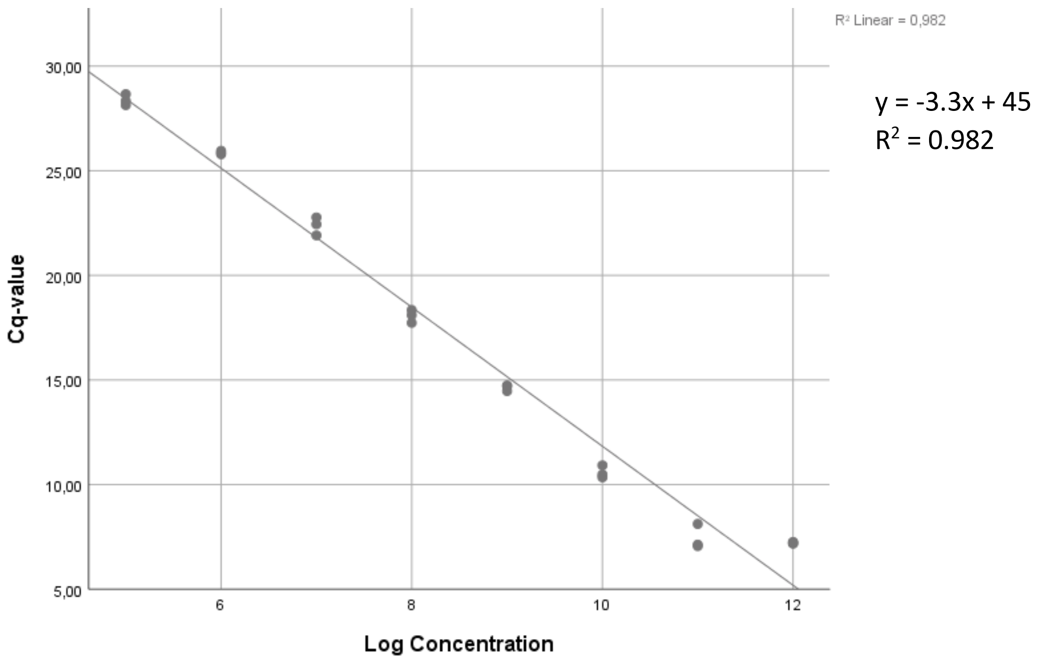


Figure 7. Standard curve of miR-223. The line represents the log concentration of 10^5 to 10^{12} and the dots represent the reported Cq-values of the synthetic miRNA miR-223.

Table 9. Average Cq-values, copies and concentration of test three samples containing miR-223 per method. *This value indicates the only measurable value, instead of average

Sample	Average Cq	Copy number	Log Concentration
Pre, 10^6	38.86*	73.42*	1,86
Pre, 10^5	35.71*	650,38*	2,81
Post, 10^6	26.80	359,512 ± 107,985	5,55
Post, 10^5	30.00	33946.88 ± 1512.088	4,53

Discussion

The project sought out to examine whether the novel two-tailed RT-qPCR method developed by TATAA Biocenter (Androvic, Valihrach, Elling, Sjoback & Kubista, 2017) was applicable in a different laborative environment than that of TATAA Biocenter. Ultimately, the goal was achieved, although a number of parameters and factors had an effect on the outcomes of the experiments.

The usage of qPCR as a technique of amplification of target DNA is a well-established method and uses either absolute quantification to measure expression levels of an absolute number of copies, or relative quantification which measure expression levels by determining fold change (Applied Biosystems, 2004). It determines these changes in samples and expresses it relative to a reference gene that are amplified together with the target gene (Applied Biosystems, 2004). For this project, absolute quantification and a standard curve was used to determine the expression levels in the spiked test samples. By using absolute quantification, it is easy to determine concentration values in samples containing unknown quantities since the values are extrapolated from the known standards. The test samples were spiked with the same synthetic miRNA as was used to construct the standard curve, however, the test samples also contained unknown quantities of miRNA naturally occurring in plasma from self-assessed healthy individuals. The standard curve method assumes that amplification efficiency is going to be the same for the standard curve samples and the test samples, which can cause varying results (Brankatschk, Bodenhausen, Zeyer, & Bürgmann 2012).

There are a wide range of options for miRNA extraction, with kits varying in the time it takes for the procedure to be performed, cost and whether or not it can be performed with machines or by hand. However, the kit used for extraction will also affect the yield and the quality of the miRNA (Wright, de Silva, Purdie & Plain, 2020). Currently, there is no standardized extraction kit for miRNA. In a study performed by Wright, de Silva, Purdie & Plain (2020), they compare different commercial miRNA isolation and extraction kits. According to the study, the miRNeasy Serum/Plasma kit (Qiagen) was the most optimal across samples and displayed little variation between fresh and frozen plasma. The kit used for this project was the miRNeasy Serum/Plasma Advanced kit (Qiagen), which Wright, de Silva, Purdie & Plain (2020) rank as second best, which had similar miRNA detection in fresh samples but less optimal for frozen samples. The miRNeasy Serum/Plasma kit (Qiagen) also has a column-based approach with the possibility to render high concentrations of miRNA without the need of extra purification steps (Moret et al, 2013). The kits PureLink RNA (Thermo Fisher Scientific) and Isolate II miRNA kit (Bioline) require less sample volume and are fast and simple, but perform poorly in detecting miRNA and show great variation across samples (Wright, de Silva, Purdie & Plain, 2020). According to another study performed by Brown, Epis, Horsham, Kabir, Richardson and Leedman (2018), the miRNeasy kit (Qiagen) is able to consistently yield large amounts of concentrated miRNA in contrast to other extraction kits. Therefore, if another miRNA extraction kit had been used for this project, the results might have differed.

Qubit microRNA assay (Life Technologies, USA) provides a kit for measuring quantity of RNA or miRNA in the assay range of 0.5 ng/µl to 100 ng/µl. When compared to other methods for this purpose such as Nanodrop (Thermo Scientific) and the Agilent 2100 Bioanalyzer, Qubit was shown to be the most consistent with results from quantification of RNA (Wright, de Silva, Purdie & Plain, 2020). Nanodrop is quick and does not rely on extra chemicals, but does not distinguish between RNA and DNA or other substances present such as phenol, which makes it a much less reliable method. Bioanalyzer is costly and time consuming, but is able to provide a detailed quality assessment of RNA integrity (Wright, de Silva, Purdie & Plain, 2020). While Qubit has high specificity and sensitivity, it does not have the ability to measure purity. Contaminants like proteins, phenol and salt are not detected (Thermo Fisher Scientific, 2020).

The Qubit fluorometer (Life Technologies) was primarily used to detect any present miRNA in the test samples. Three samples were prepared for each of the four categories (Figure 3) in order to gain variety and to optimize the procedure, and to have more options if a sample's concentration was too low to be detected. The samples that had the highest concentration in each group were chosen for reverse transcription and qPCR. However, the mean concentrations of all the measured samples do not show a great variation (Table 5 and 6) rather, they all show to be within the range of 0,500-0,999 ng/µl. This is to be expected, since the use of exogenous controls or spike-in controls in the form of synthetic miRNA, will reduce variability between samples (Kroh, Parkin, Mitchell, & Tewari, 2010). It is also worth noting that Qubit microRNA assay (Thermo Fisher Scientific) is able to detect miRNA in a range of 0.5 ng/µl to 100 ng/µl. In other words, the samples that were measured in the Qubit displayed a concentration on the absolute lower end, so higher concentrations closer to 100 ng/µl might have given different results in purity.

A common consensus is that the 260/280 ratio and the 260/230 ratio, which is a measure of purity of the samples, of DNA or RNA measured with the NanoDrop spectrophotometer should fall within the range of 1.8-2.0 (Thermo Fisher Scientific). However, NanoDrop might not be suitable for quantification and measurement of purity of extracted miRNA, mostly due to its small size, because nucleic acid precipitation, a process in which DNA or RNA is concentrated, is less effective for small nucleic acids than longer nucleic acids (Kim et al., 2012). Tiberio, Callari, Angeloni, Daidone and Appierto (2015) describe the similar view; miRNA is undetectable when using NanoDrop. It does, however, measure total RNA, and findings by El-Khoury et al. (2016) suggest that the purity measured at a 260/280 wavelength is actually dependent on the starting volume of the extraction procedure, since a higher starting volume has a better capability to give a higher concentration. Across different extraction kits such as the miRneasy kit (Qiagen), miRCURY RNA Isolation kit (Exiqon) and TRIzol RNA Isolation kit (Invitrogen), each kit showed a purer reading with increasing starting volume, with the purest reading of 1.96 for the volume of 91.2 ng/µl for the miRNeasy kit (Qiagen). The results from the extractions performed in this thesis had a much lower concentration and starting volumes (Table 5 and 6) which could be a factor for the generally impure readings (Table 7 and 8). The miRNeasy Serum/Plasma Advanced kit allows for up to 600 µl to be extracted at a time, but is not recommended, due to the specificity of the extraction process being reliant on the even distribution within the spin column (Qiagen handbook). A common contaminant is phenol, which miRNeasy Serum/Plasma Advanced kit (Qiagen) has excluded in this kit, in comparison to Qiagen's older miRNA extraction kit.

Another important aspect to be taken into consideration is the possibility of altering miRNA levels by hemolysis. Hemolysis is the disruption of red blood cells (RBC) and their release of hemoglobin into the surrounding media, which can occur due to inappropriate handling of blood samples and inappropriate storage conditions, and even abnormalities in the blood of the donor (Sowemimo-Coker, 2002). Studies have shown that the hemolysis occurred during blood collection can have a substantial impact on the miRNA content in biofluids such as plasma and serum. Kirschner et al (2013) show in their study that a large number of miRNAs are indeed affected by hemolysis, and miR-let7b that was used in this project was one of them. Currently, hemolysis is not always a factor that is considered when analyzing miRNA in serum or plasma (Kirschner et al, 2013).

A reason for measuring the purity is to be able to assess the values later on given by the RT-qPCR, a process in which potential contaminations could have occurred. The miRNA extracted from plasma spiked with miR-let7b showed to be the closest to the desired value of 2.0, but is suspected to have been contaminated as explained further below.

To optimize the method of the two-tailed RT-qPCR, absolute quantification is used to determine expression levels and this is achieved with the help of a standard curve that is implemented in the RT-qPCR together with the samples. The results from the RT-qPCR show a similar cycle quantification for miR-223 both for test samples (Table 9) and the standard curve (Figure 7). This suggests that the optimized methodology can be used for future research. For miR-let7b the results showed positive results in the negative controls, and the R^2-value of the standard curve was very far from the desired >0.990 or >0.980 (Taylor, Wakem, Dijkman, Alsarraj, & Nguyen 2015). Similar results were acquired even when repeating the experiment with newly diluted primers (data not shown). The same reagents were used for miR-223 for the reverse transcription and qPCR (with the exception of miRNA specific primers), and miR-223 did not show contamination, as well as a R^2-value of >.980. The amplification efficiency of the test samples spiked with synthetic miRNA miR-223 was 100% (Figure 7). This could suggest that the samples as well as the synthetic miRNA for miR-let7b that were used for spike-in were contaminated. Life Technologies (2010) suggests that if amplification is shown in the no reverse transcriptase controls, the extraction method could be at fault, as well as the reagents or the samples. Although, if the difference between the Cq-values from the no reverse transcription reactions and the Cq-values from the sample is greater than 5, the sample is ok and this can be further investigated by doing a melt curve analysis which shows if there are multiple peaks, which then would indicate contamination (Bio-Rad, n.d.). Additionally, the reagents for reverse transcription and qPCR were used simultaneously by another person which also increases the risk of contamination.

The slope for the standard curve should fall within the range of -3.3 and -3.8, which was achieved by miR-223. Although yet again, not achieved by miR-let7b (data not shown). The slope indicates the efficiency and should be within 90-100%. The samples that were spiked with miR-223 had an efficiency of approximately 100%, and the samples that were spiked with miR-let7b reported an efficiency of 129%. The reason for achieving a greater efficiency than 100% is most likely due to polymerase inhibition, which is caused by contaminants in the sample, most likely from the extraction procedure (Kralik & Ricchi, 2017). These inhibitors will cause the Cq-values to decrease to a value lower than -3.3, and then back up to -3.3 in a more diluted sample point where there is less inhibition. This will flatten out the efficiency plot giving a lower slope which results in an efficiency that exceeds 100% (BioSistemika, 2017). However, if there is a consistent difference between Cq-values between the standard points, but the efficiency is still greater than 100%, a consistent pipetting error could be at fault rather than polymerase inhibition (Applied Biosystems, 2004). If the sample DNA or RNA used is contaminated, it is possible to purify it with DNase or RNase. Such a component was however not included in the miRNeasy Serum/Plasma Advanced kit (Qiagen), which is why it was not considered in this project.

A statistical analysis was deemed unnecessary given the variables that were used in the project. Limitations of low sample size were due to time constraints and access limitations to the lab. The sample size consisting of only three samples per synthetic miRNA, extraction method and copy number, is not enough to conduct trustworthy statistics with, since a larger sample group is generally preferred for statistics (Sandelowski, 1995). Graphs are instead used to depict differences of the methods and miRNA. For example, the samples that were spiked with synthetic miRNA after miRNA extraction should contain a higher amount of miRNA, since miRNA extraction can degrade or compromise the miRNA (Brown et al., 2018). Therefore, these post-spiked samples would have a higher concentration post amplification. This is visible in the graph (Figure 4), so one could assume the assumption is correct. Interestingly, the concentration of the post-spiked samples has a lower average concentration than the pre-spiked samples before the RT-qPCR. The higher concentration in the later stage of the process could suggest an off-target amplification target or contamination (New England Biolabs, n.d.).

The melting curve analysis for samples spiked with miR-223 (data not shown) showed a single peak which indicates a single product has been amplified. The samples that had been spiked with miR-let7b showed multiple peaks (data not shown). This could indicate that more than one DNA product was amplified, which is another factor indicating the presence of contamination or off-target amplification targets (New England Biolabs, n.d.). Pure products can produce more than one amplicon, and a gel electrophoresis could have been performed to determine this (Integrated DNA technologies, 2014, 2016). It was, however, not performed due to time constraints.

The study of miRNAs as biomarkers for diagnosis of sepsis are very promising. By using the two-tailed RT-qPCR method, this project has shown that it can be possible to determine which miRNA are up- or downregulated. MiR-223, a miRNA which has been used in the project, has since previously been shown to have lower levels of expression in serum derived from septic patients, by the help of RT-qPCR (Wang et al, 2010). With the introduction of the two-tailed RT-qPCR, which has the advantage of higher sensitivity, could eventually lead to a very secure diagnostic tool. This will in turn have a great societal and ethical impact, since the rate of mortality of sepsis and septic shock is as high as 38% (Vincent, Jones, David, Olariu & Campbell, 2019). For the people who survive sepsis or a septic shock, regardless of its severity, have a mortality rate ranging from 7% to 43% (Gauer, 2013). The current golden standard of diagnosing sepsis is the measuring of C-reactive protein and procalcitonin as well as culturing bacteria in growth media; these biomarkers, however, are not specific for sepsis. The combination of these biomarkers together with monitoring the regulation of certain miRNA using the two-tailed RT-qPCR has the promise to become an optimized method and perhaps a part of a new golden standard.

Conclusion

Conducting a RT-qPCR experiment by hand can be complicated, especially when needing to be very precise during numerous pipetting procedures. Yet, even if one of the miRNAs studied in this experiment showed less than ideal results, it is possible to conclude that the two-tailed RT-qPCR method works with detecting and analysing extracted miRNA from human plasma. Further research and experimenting to advance the idea of miRNAs as reliable biomarkers for sepsis holds great promise for decreased mortality and prolonged longevity and health.

Acknowledgements

First and foremost, I would like to thank my supervisor Anna-Karin Pernestig, for giving me a chance to partake in the project “Future diagnostics of sepsis”. The help and guidance I have received during this process has been very valuable. I would also like to thank my supervisor Sanja Jurcevic, for being a constant help and advice during this challenging task. Further, I would like to thank my examiner Magnus Fagerlind for the revisions to make my bachelor thesis the best it could be. Lastly, I would like to thank my partner, friends and family for their never-ending support and faith in me.

References

Alles, J., Fehlmann, T., Fischer, U., Backes, C., Galata, V., Minet, M., … Meese, E. (2019). An estimate of the total number of true human miRNAs. *Nucleic Acids Research*, *47*(7), 3353–3365. doi: 10.1093/nar/gkz097

Androvic, P., Valihrach, L., Elling, J., Sjoback, R., & Kubista, M. (2017). Two-tailed RT-qPCR: a novel method for highly accurate miRNA quantification. *Nucleic Acids Research*, *45*(15), e144-e144. doi: 10.1093/nar/gkx588

Applied Biosystems by Life Technologies. (2004). Guide to Performing Relative Quantification of Gene Expression using Real-Time Quantitative PCR. Retrieved 2020-05-19 from https://www.gu.se/digitalAssets/1125/1125331_ABI_-_Guide_Relative_Quantification_using_realtime_PCR.pdf

Applied Biosystems by Life Technologies. (2010). TaqMan Gene Expression Analysis Protocol. Retrieved 2020-04-16 from https://tools.thermofisher.com/content/sfs/manuals/cms_041280.pdf

Bio-Rad Laboratories Inc. (2015). A Practical Approach to RT-qPCR – Publishing Data That Conform to the MIQE Guidelines. Bio-Rad Bulletin 5859 Ver E. Retrieved 2020-04-22 from http://www.bio-rad.com/webroot/web/pdf/lsr/literature/Bulletin_5859.pdf

Brankatschk, R., Bodenhausen, N., Zeyer, J., & Bürgmann, H. (2012). Simple Absolute Quantification Method for Quantitative PCR Efficiency Variations for Microbial Community Samples. *Applied and Environmental Microbiology, 78*(12), 4481-4489. doi:10.1128/aem.07878-11

Brown, R., Epis, M., Horsham, J., Kabir, T., Richardson, K., & Leedman, P. (2018). Total RNA Extraction from Tissues for MicroRNA and Target Gene Expression Analysis: Not all kits are created equal. *BMC Biotechnology, 18*(16). doi:10.1186/s12896-018-0421-6

Correia, C. N., Nalpas, N. C., McLoughlin, K. E., Browne, J. A., Gordon, S. V., MacHugh, D. E., & Shaughnessy, R. G. (2017). Circulating microRNAs as Potential Biomarkers of Infectious Disease. *Frontiers in Immunology*, *8*. doi: 10.3389/fimmu.2017.00118

Deng, R., Zhang, K., & Li, J. (2017). Isothermal Amplification for MicroRNA Detection: From the Test Tube to the Cell. *Accounts of Chemical Research*, *50*(4), 1059-1068. doi: 10.1021/acs.accounts.7b00040

El-Khoury, V., Pierson, S., Kaoma, T., Bernardin, F., & Berchem, G. (2016). Assessing Cellular and circulating miRNA Recovery: The Impact of the RNA Isolation Method and the Quantity of Input Material. *Sci. Rep*, *6*(19529). doi:10.1038/srep19529

Faraldi, M., Gomarasca, M., Sansoni, V., Perego, S., Banfi, G., & Lombardi, G. (2019). Normalization strategies differently affect circulating miRNA profile associated with the training status. *Scientific Reports*, *9*(1). doi: 10.1038/s41598-019-38505-x

Filipowicz, W., Bhattacharyya, S. N., & Sonenberg, N. (2008). Mechanisms of post-transcriptional regulation by microRNAs: are the answers in sight? *Nature Reviews Genetics*, *9*(2), 102-114. doi: 10.1038/nrg2290

Gauer, R. (2013). Early Recognition and Management of Sepsis in Adults: The First Six Hours. *Am fam Physician*, *88*(1), 44-53. Retrieved 2020-05-08 from https://www.aafp.org/afp/2013/0701/p44.html

IBM Corporation. (2017). IBM SPSS Statistics for Windows (version 25). [Computer software] Armonk, N.Y., USA

Karthikeyan, G., & Premkumar, K. (2001). Neonatal Sepsis: Staphylococcus aureus as the predominant pathogen. *The Indian Journal of Pediatrics*, *68*(8), 715–717. doi: 10.1007/bf02752407

Kim, Y.-K., YEO, J., Kim, B., Ha, M., & Kim, V. N. (2012). Short Structured RNAs with Low GC Concent Are Selectively Lost during Extraction from a Small Number of Cells. *Molecular Cell*, *46*(6), 893-895. https://doi.org/10.1016/j.molcel.2012.05.036

Kingsley, S. M. K., & Bhat, B. V (2017). Role of microRNAs in sepsis. *Inflammation Research*, *66*(7), 553-569. doi:10.1007/s00011-017-1031-9

Kirschner, M., Edelman, J., Kao, S., Vallely, M., van Zandwijk, N., & Reid, G. (2013). The Impact of Hemolysis on Cell-Free microRNA Biomarkers. *Frontiers in Genetics*, *4*. doi: 10.3389/fgene.2013.00094

Kirschner, M., van Zandwijk, N., & Reid, G. (2013). Cell-free microRNAs: Potential Biomarkers in need of Standardized Reporting. *Frontiers in Genetics*, *4*(56). doi:10.3389/fgene.2013.00056

Kralik, P., & Ricchi, M. (2017). A Basic Guide to Real Time PCR in Microbial Diagnostics: Definitions, Parameters, and Everything. *Frontiers in Microbiology*, *8*. doi:10.3389/fmicb.2017.00108

Kroh, E. M., Parkin, R. K., Mitchell, P. S., & Tewari, M. (2010). Analysis of circulating microRNA biomarkers in plasma and serum using quantitative reverse transcription-PCR (qRT-PCR). *Methods*, *50*(4), 298–301. doi:10.1016/j.ymeth.2010.01.032

Lambregts, M. M., Bernards, A. T., van der Beek, M. T., Visser, L. G., & de Boer, M. G. (2019). Time to positivity of blood cultures supports early re-evaluation of empiric broad-spectrum antimicrobial therapy. *PLOS ONE*, *14*(1). doi: 10.1371/journal.pone.0208819

Ljungström, L., Pernestig, A.-K., Jacobsson, G., Andersson, R., Usener, B., & Tilevik, D. (2017b). Diagnostic accuracy of procalcitonin, neutrophil-lymphocyte count ratio, C-reactive protein, and lactate in patients with suspected bacterial sepsis. *PLOS ONE*, *12*(7). doi: 10.1371/journal.pone.0181704

Moret, I., Sánchez-Izquierdo, D., Iborra, M., Tortosa, L., Navarro-Puche, A., Pilar., ... Beltrán, B. (2013). Assessing an Improved Protocol for Plasma microRNA Extraction. *PLoS ONE* 8(12): e82753. doi: 10.1371/journal.pone.0082753

New England Biolabs. (n.d.). Dye-based qPCR & RT-qPCR. Retrieved 2020-05-19 from https://international.neb.com/applications/dna-amplification-pcr-and-qpcr/qpcr-and-rt-qpcr/dye-based-qpcr

New England Biolabs. (n.d.). Reverse Transcription (cDNA Synthesis). Retrieved 2020-05-19 from https://international.neb.com/applications/cloning-and-synthetic-biology/dna-preparation/reverse-transcription-cdna-synthesis

New England Biolabs. (n.d.). qPCR & RT-qPCR. Retrieved 2020-05-19 from https://international.neb.com/applications/dna-amplification-pcr-and-qpcr/qpcr-and-rt-qpcr

Qubit Fluorometric Quantitation: Thermo Fisher Scientific - US. (n.d.). Retrieved 2020-03-20 from https://www.thermofisher.com/se/en/home/industrial/spectroscopy-elemental-isotope-analysis/molecular-spectroscopy/fluorometers/qubit.html

Sandelowski, M. (1995). Sample Size in Qualitative Research. *Research in Nursing & Health*, *18*(2), 179-183. doi:10.1002/nur.4770180211

Sowemimo-Coker, S. (2002). Red Blood Cell Hemolysis during Processing. *Transfusion Medicine Reviews*, *16*(1), 46-60. doi:10.1053/tmrv.2002.29404

Plasma and Serum Preparation. (2007). Retrieved 2020-03-20 from https://www.thermofisher.com/se/en/home/references/protocols/cell-and-tissue-analysis/elisa-protocol/elisa-sample-preparation-protocols/plasma-and-serum-preparation.html

Pogribny, I. P. (2017). MicroRNAs as biomarkers for clinical studies. *Experimental Biology and Medicine*, *243*(3), 283–290. doi:10.1177/1535370217731291

Vasilescu, C., Rossi, S., Shimizu, M., Tudor, S., Veronese, A., Ferracin, M., … Calin, G. (2009). MicroRNA Fingerprints Identify miR-150 as a Plasma Prognostic Marker in Patients with Sepsis. *PloS One*, *4*(10). doi:10.1371/journal.pone.0007405

Vincent, J.-L, Jones, G., David, S., Olariu, E., & Cadwell, K. (2019). Frequency and Mortality of Septic Shock in Europe and North America: A systematic review and meta-analysis. *Critical Care, 23*(196). doi:10.1186/s13054-019-2478-6

Vincent, J.-L., de Mendonca, A., Cantraine, F., Moreno, R., Takala, J., Suter, P. M., … Blecher, S. (1998). Use of the SOFA score to assess the incidence of organ dysfunction/failure in intensive care units. *Critical Care Medicine*, *26*(11), 1793–1800. doi:10.1097/00003246-199811000-00016

Wang, J., Yu, M., Yu, G., Bian, J., Deng, X., Wan, X., & Zhu, K. (2010). Serum miR-146a and miR-223 as Potential New Biomarkers for Sepsis. *Biochemical and Biophysical Research Communications, 394*(1), 184-188. doi:10.1016/j.bbrc.2010.02.145

Wright, K., de Silva, K., Purdie, A., & Plain, K. (2020). Comparison of methods for miRNA isolation and quantification from ovine plasma. *Scientific Reports*, *10*(1). doi:10.1038/s41598-020-57659-7

Appendices

Appendix 1: Samples of miRNA miR-223

Qubit and NanoDrop results for pre- and post-spiked miR-223 samples.

Sample	miRNA concentration	miRNA purity (260/280 absorbance value)
Pre, 10^6	0,760 ng/µl	1.20
Pre, 10^6	0,840 ng/µl	0.73
Pre, 10^6	0,600 ng/µl	1.35
Pre, 10^5	0,960 ng/µl	0.46
Pre, 10^5	Out of range	1.55
Pre, 10^5	0,680 ng/µl	0.21
Post, 10^6	0,860 ng/µl	1.19
Post, 10^6	0,780 ng/µl	0.96
Post, 10^6	0,880 ng/µl	1.55
Post, 10^5	0,620 ng/µl	1.15
Post, 10^5	0,620 ng/µl	1.27
Post, 10^5	0,540 ng/µl	1.25

Appendix 2: Samples of miRNA miR-let7b

Qubit and Nanodrop results for pre- and post-spiked miR-let7b samples.

Sample	miRNA concentration	miRNA purity (260/280 absorbance value)
Pre, 10^6	0,900 ng/µl	1.60
Pre, 10^6	0,860 ng/µl	1.47
Pre, 10^6	1,22 ng/µl	1.50
Pre, 10^5	0,760 ng/µl	0.80
Pre, 10^5	0,700 ng/µl	1.03
Pre, 10^5	0,860 ng/µl	0.95
Post, 10^6	0,700 ng/µl	2.70
Post, 10^6	0,720 ng/µl	1.44
Post, 10^6	0,640 ng/µl	1.35
Post, 10^5	0,600 ng/µl	1.33
Post, 10^5	0,700 ng/µl	1.30
Post, 10^5	0,660 ng/µl	1.35