Degree project

UNIVERSITY OF SKÖVDE

Manual and robotic RNA extraction from human plasma with absolute quantification of miRNA through two-tailed RT-qPCR as part of research into early diagnosis of sepsis.

Bachelor Degree Project in Bioscience

First Cycle 30 credits

Spring term 2022

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Abbreviations

AGO – Argonaut

AUC – Area under the curve

cDNA – Complementary deoxyribonucleic acid

Cq – Quantitation cycle

CRP – C-reactive protein

DNA – Deoxyribonucleic acid

dsDNA – Double-stranded deoxyribonucleic acid

IQR – Interquartile range

JAMA – American Medical Association

LOD – limit of detection

LOQ – limit of quantification

miRNA – Micro-ribonucleic acid

NSA – Non-spiked automated extraction

NSM –Non-spiked manual extraction

PCT – Procalcitonin

qPCR – Quantitative polymerase chain reaction

RCF – Relative centrifugal force

RNA – Ribonucleic acid

RT – Reverse transcription

SA – Spiked automated

SD – Standard deviation

SM – Spiked manual extraction

SOFA – Sequential organ failure assessment

ssRNA – Single stranded Ribonucleic acid

TTD – Time to diagnosis

Abstract

Each hour´s delay in administering antibiotics has been shown to result in a 9% increase in the odds of mortality in sepsis cases. It is thus evident that the development of a diagnostic method that ensures an early time to diagnosis of sepsis is essential. MiRNAs have shown promise with regards to diagnostic capabilities concerning sepsis, with differential expression of circulatory miRNAs seen during various diseased states. MiRNA can be quantified directly from a blood plasma sample, greatly decreasing the time to diagnosis, as the requirement for culturing is eliminated. Quantification of miRNA by means of qPCR has proven rather challenging, due to their short length. A solution might be two-tailed RT-qPCR, a method which utilizes a two-tailed RT primer. The aim of the project was to optimize the extraction and quantification of miRNAs from minimal amounts of human blood plasma samples, as to create a standardized and reproduceable method for measuring biomarker miRNAs within human blood plasma. In this study, a significant difference between manual and semi-automated extraction of miRNA from plasma with regards to A260/A280 ratios ($p = 0.00$) was observed. It was also found that a correlation exists between A260/A280 ratios and miR-seps6 quantified, using the two-tailed RT-qPCR method. This method has shown to be effective at amplifying circulating miR-seps6 arising from 100 µL of human blood plasma. A linear standard curve, constructed from synthetic miR-seps 6 produced optimal amplification efficiencies, and the melt curve indicated a single product, which correlates with good specificity. As successful detection and amplification of miR-seps 6 had been achieved during this study, the next phase of the project can be initiated, where it will be attempted to detect miR-seps6 from plasma stored in a human biological material bank (biobank).

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Introduction

Sepsis has been mentioned since the time of the ancient Greeks, both in the poems of Homer, and in the medical texts written by Hippocrates, where "biological decay" was believed to be the cause of the syndrome (Funk et al., 2009). More recently, with the discovery of microorganisms, a link was made between infection and sepsis, where infection was a cause for the host reaction, that leads to the occurrence of sepsis (Rittirsch et al., 2008). In 2019, a study by Ljungström et al. (2019) concluded that the incidence rate of sepsis-3 in Sweden was 838/100000 (95% CI: 798 – 877), with persons living with comorbidities more likely to contract severe sepsis. Sepsis is also costly, with an estimated 6.2 % of all hospitalization costs in the United States of America being attributed to sepsis (Fleischmann-Struzek et al., 2020).

The onset of sepsis occurs when the host´s immune response to a pathogen result in damage to its own tissues (Eubank et al., 2020). Infection by a wide variety of pathogens can bring about sepsis. These include Gram-negative bacteria, Gram-positive bacteria, viruses, and fungi (Jean-Baptiste, 2007), with the most common aggressor being varied, depending on the geographical location (Cheng et al., 2008). Sepsis can be caused by intrinsic factors, as well as extrinsic factors (Jean-Baptiste, 2007). Extrinsic factors include the presence of molecules because of infection. Intrinsic factors are those caused by trauma, resulting in a response by the host immune system, which causes the release of proinflammatory mediators such as tumour necrosis factor alpha, interleukin 1, interleukin 6, and high-mobility group box-1 protein to name a few (Jean-Baptiste, 2007; Ono et al., 2018).

According to the American Medical association (JAMA), Sepsis is defined as "life threatening organ dysfunction caused by a dysregulated host response to infection" (Singer et al., 2016). Sepsis-3 suggests increased risk of a poor outcome and is defined as presenting with two of the following three variables: Low blood pressure (≤ 100 mmHg), high respiratory rate (≥ 22 breaths per minute) and altered mentation (<15 according to the Glasgow coma scale) (Singer et al., 2016).

Sepsis is typically diagnosed by obtaining two blood cultures, one being aerobic and the other being anaerobic as to identify the pathogen responsible (Rhodes et al., 2017). With conventional automated culturing yielding varying times to detection (TTD), ranging anywhere from > 10 hours up to > 80 hours, depending on the pathogen in question (Viganò et al., 2002). Improvements have been made in terms of the TTD, through the utilization of novel technologies, such as quantitative polymerase chain reaction (qPCR), and matrix-assisted laser desorption ionization time-of-flight (MALDI-TOF) and mass-spectrometry (MS), where waiting times decreased significantly, to around an hour, such as with MALDI-TOF and qPCR (Eubank et al., 2020). However, Eubank et al. (2020) mentions that independently, these technologies have not proven to be effective at diagnosing sepsis. These diagnostic efforts have been focused on the identification of pathogens in the patient, which explains the lack in specificity, as the presence of a pathogen does not infer sepsis. To aid the diagnosis of sepsis, the "Sequential organ Failure Assessment" (SOFA) is employed, which considers a variety of biomarkers associated with organ failure and creates a score of between 1 and 4, where a higher score is associated with a higher mortality rate (Singer et al., 2016). Along with SOFA, other biomarkers, such as c-reactive protein (CRP) and procalcitonin (PCT) are typically used to aid in the diagnosis of sepsis. PCT and CRP have either not shown sufficient specificity or sensitivity in diagnosing sepsis (Tsalik et al., 2012).

Liu et al. (2017) found that each hour´s delay in administering antibiotics has shown to result in a 9% increase in the odds of mortality in sepsis cases. It is thus evident that the development of a diagnostic method that ensures a quick TTD of sepsis is essential. To find such a diagnostic tool, which is also high in specificity, as well as sensitivity, a multivariate approach could be considered.

The inclusion of microRNAs (miRNAs) in a biomarker panel might yield a high specificity and sensitivity for sepsis, whilst decreasing the TTD. MiRNAs have shown promise with regards to diagnostic capabilities concerning sepsis, with differential expression of circulatory miRNAs seen during various diseased states (Yao et al., 2015; Busk, 2014). Due to the presence of circulating miRNA in blood plasma (Yao et al., 2015), miRNA can be quantified directly from a blood plasma sample, greatly decreasing the TTD, as the requirement for culturing is eliminated (Essandoh & Fan, 2014). Wu et al. (2013) showed that miRNA in the blood serum of sepsis induced mice was upregulated, with certain miRNAs showing between 50- and 100-fold upregulation 24 hours after cecal ligation and puncture. A study by Yao et al. (2015) compared the clinical accuracy of miRNA-25, a circulating miRNA, to that of CRP and PCT, which resulted in a higher area under ROC curve (AUC) for miRNA-25 (AUC= 0.806; 95% CI: 0.701 – 0.912) than that of CRP (AUC= 0.676; 95% CI: 0.54 – 0.810) and PCT (AUC= 0.726; 95% CI: 0.592 – 0.860) ($p<0.05$).

The biogenesis of miRNA starts with the transcription of pri-miRNA by polymerase II or polymerase III. The pri-miRNA is then modified by the RNase III DROSA-DGCR8 (Han et al., 2004), resulting in pre-miRNA, which is exported from the nucleus to the cytoplasm. In the cytoplasm, the double-stranded pre-miRNA molecule is unwound by a RNase III discar protein, resulting in two single-stranded miRNAs, of which one is typically degraded, however, both stands can be processed into mature miRNA (Essandoh & Fan, 2014). The remaining miRNA has two pathways, where one results in the release of the molecule from the cytoplasm to the extracellular space, and the other results in the integration of the miRNA into an RNA-induced silencing complex (RISC) complex, which is composed primarily of an Argonaut (AGO) protein and the miRNA (Pratt & MacRae, 2009) and targets messenger RNA (mRNA). Binding of the RISK complex to a target mRNA results in either silencing or degradation of the mRNA (Benz et al., 2016), due to the RNase activity of the AGO protein (Pratt & MacRae, 2009).

Most miRNAs are present within cellular tissue, but many have been detected in various types of bio-fluid, and are known as circulatory miRNAs (Sohel, 2016). Circulating miRNA have shown stable expression in bio-fluids, even when subjected to harsh conditions such as high pH and boiling (Essandoh & Fan, 2014; Sohel, 2016). Cellular miRNAs on the other hand tend to degrade quickly in biofluids such as plasma (Arroyo et al., 2011; Sohel, 2016), which is likely due to the presence of RNases. It has been hypothesised that protection of circulating miRNA against RNase activity is afforded through encapsulation within exosomes, integration into an AGO ribonucleoprotein complex, or a combination of both (Arroyo et al., 2011).

Extraction of circulatory miRNA from a human plasma can be challenging due to various factors, such as the low concentrations typically present within blood plasma (Andreasen et al., 2010), cellular contamination, and inhibitors that result from the purification process (Androvic et al., 2019). Androvic et al. (2019) reported that a non-linear relationship exists between an increase in sample volume and the amount of miRNA detected, which indicates that an increase in the starting amount of blood plasma increases the amount of cellular material and inhibitors as which might negatively affect circulatory miRNA quantification. It is thus important that an optimal amount of starting material is used. Due to the challenges pertaining to extraction of miRNA from difficult bio-fluids such as blood plasma, it is expected that hands-on time and consequently, TTD is adversely affected. A standardized extraction method is thus required for circulating miRNAs to be utilized as diagnostic and prognostic biomarkers, with particular emphasis placed on lowering TTD and technical variation. A solution in the form of a semi-automated system might resolve some of these issues. The QIAcube® is a semi-automated machine capable of extracting nucleic acids from 12 samples simultaneously (Marzi et al., 2016; Sharma et al., 2022). It typically takes around 10 minutes to set up the reaction, dramatically decreasing the hands-on time of extracting nucleic acid, as compared to manual extraction (Sharma et al., 2022). The QIAcube®,

however, has its limitations, such as a maximum centrifugations speed of 12 000 g (QIAcube® user manual, 2018), and set volumes with regards to assumed starting volumes, which might result in suboptimal reagent amounts used during QIAcube® extraction.

Various miRNA quantification methods exist, such as northern blotting, *in situ* hybridization, next-generation sequencing, and microarrays (Androvic et al., 2017; Wang et al., 2012; Yang et al., 2014). These methods have proven inefficient for clinical use as they are either too complex, expensive, or not specific enough (Androvic et al., 2017; Wang et al., 2012; Yang et al., 2014). Quantitative polymerase chain reaction (qPCR), however, provides a solution to this, as it is relatively inexpensive, precise, and relatively quick to perform (Yang et al., 2014). Quantification of miRNA by means of qPCR has proven rather challenging, due to their short length (19-24 nt), which similar in length to a typical primer, limiting the ability of such primers to be utilized, as overlapping of said primers would occur (Androvic et al., 2017; Busk, 2014). It is thus required to increase the length of the miRNA template during the reverse transcription stage, to have a template long enough for forward and reverse primers to be effective during the qPCR stage (Forero et al., 2019).

There exist two fundamental reverse transcription techniques, namely, universal, and specific reverse transcription (Androvic et al., 2017). An example of universal reverse transcription is the poly (T) adapter strategy, which consists of first poly adenylating the mature miRNA, followed by the addition of a poly (T) adapter, which allows for first strand cDNA synthesis. A target specific forward primer and adapter specific reverse primer is then utilized (Shi & Chiang, 2005). Specific reverse transcription refers to methods that aim to only reverse transcribe target sequences and as such rely on a reverse transcription primer that is specific to the target sequence, and hence results in the reverse transcription of the sequence in question (Forero et al., 2019), of which an example is the stem-loop primer method. Stem-loop primers consist of a hairpin structure, and a 3´ binding region. The stem loop structure present within the primer results in favouring RNA/DNA duplex formation with ssRNA (Androvic et al., 2017) and thus avoiding annealing to pri and pre-miRNA, as these species contain a stem-loop (Pratt and MacRae, 2009).

Two-tailed RT-qPCR, a method described by Androvic et al. (2017), utilizes a two-tailed RT primer that is similar to a traditional stem-loop primer in that it contains a stem loop, with a 3´ probe that is specific to the target. What differentiates the two-tailed RT-qPCR method from other stem-loop based methods is the presence of a longer 5´ hemiprobe, which is specific to a different region than the 3´ hemiprobe within a specific miRNA target. After binding to the target molecule, the reverse transcriptase initiates cDNA synthesis from the 3´ of the hemiprobe, and as synthesis continues, displaces the 5´ hemiprobe, resulting in a cDNA composed of the two-tailed RT primer with the target sequence integrated on its 3´. qPCR is then applied, using SYBR green, which is an intercalating dye (Ye et al., 2019). During qPCR, the forward primer binds to the ´5 of the cDNA, and the reverse primer binds to the 3´, which contains the target sequence (Androvic et al., 2017).

Androvic et al. (2017) compared the two-tailed RT-qPCR method to the TaqMan microRNA assay, which uses a traditional stem-loop primer with only a 3´-probe specific to the target and found that the two-tailed RT-qPCR method is superior in terms of specificity, with < 1% signals observed from assays containing mismatched targets, as compared to the TaqMan microRNA assay, which resulted in 22.48% signals observed from assays containing mismatched targets. With regards to the two-tailed RT-qPCR method, all the false positive signals observed originated from assays that contained molecules that differed by only one nucleotide from the target miRNA. These results indicate the suitability of the two-tailed RT primer for use in diagnostics, as it could discriminate between miRNAs with minor variations (Androvic et al., 2017).

Two quantification methods are typically used to determine the starting amount of template within a qPCR reaction, namely, relative quantification and absolute quantification (Livak & Schmittgen, 2001). Relative quantification makes use of a reference gene, where a reference gene is expected remain consistent regardless a variation of expression in the transcriptome (Whelan et al., 2003). The target template can then be compared to the reference gene to determine whether the template is differentially expressed and to what degree through using the $2^{-\Delta\Delta ct}$ method (Livak & Schmittgen, 2001). Absolute quantification on the other hand, makes use of a linear standard cure, which is typically created by serially diluting the template and subjecting the varying concentrations to qPCR (Svec et al., 2015). Concentrations of samples of unknown concentration can then be determined through linear regression (Svec et al., 2015). The linear standard curve also allows for the determination of both reaction efficiency and coefficient of determinant (Svec et al., 2015).

Research question

Two research questions are to be explored during this project. Firstly, does two-tailed RT-qPCR enable specific detection and consistent quantification of miR-seps 6 extracted from human blood plasma when optimized? MiRNAs may demonstrate variations in behaviour due to differences in physiochemical properties (Redshaw et al., 2013), and as such, may act differently to other miRNAs during miRNA extraction, cDNA synthesis and qPCR. Previous success in detecting biomarker miRNAs other than miR-seps 6 can thus not be relied upon when determining whether a certain method has the ability to extract and quantify miR-seps 6 (Schwaber et al., 2019). A study by Androvic et al. (2017) on two-tailed RT-qPCR yielded promising results, with high reproducibility, sensitivity, and specificity. It is thus hypothesised that the two-tailed RT-qPCR experiments during this project will yield similar results. Secondly, does semi-automated extraction with QIAcube® result in a more consistent purity and miR-seps 6 yield as compared to manual extraction, where the miRNeasy advanced kit, and a 100 µl plasma starting volume is used? Semi-automated extraction could decrease the effects of handling bias and as such, may deliver a more consistent result in comparison to manual extraction (Sharma et al., 2022). It is, however, uncertain whether the extraction parameters above is optimized for miR-seps 6. To determine differences in extraction efficiency, miRNAs will be extracted using both manual and semi-automated means, quantified through qPCR, and compared to one another scope whether there is a significant difference between the two means of extraction. These questions are relevant, as the "future diagnostics of sepsis" research group at the University of Skövde intends to create a diagnostic kit to aid in the early diagnosis of sepsis where efficient, reproducible, and accurate results are vital for a successful diagnostic kit.

Aim and objectives

Due to the substantial diagnostic potential of miRNAs as biomarkers with regards to sepsis, methods for the extraction and quantification of miRNAs that deliver consistent and timely results needs to be developed and assessed, as to ensure the effectiveness of said miRNAs if included in a multi-marker panel designed to diagnose sepsis. The aim of the project was to optimize the extraction and quantification of miRNAs from minimal amounts of human blood plasma samples, as to create a standardized and reproduceable method for measuring biomarker miRNAs within human blood plasma. Extraction efficiency, amplification efficiency, and presence of amplification of natural circulating miRNA along with reproducibility of the method was considered to discern whether the aim is achieved.

To achieve the aim, certain objectives have been set out. Firstly, extracting whole RNA from 100 µL human blood plasma both manually and semi-automatically, using a QIAcube®. The manual and semi-automatically extracted eluates should then be compared in terms of consistency with

regards to RNA yield and purity. Optimization of the two-tailed RT-qPCR assay should then be performed, using a known miRNA as template, namely miR-223. Mi-223 makes a good candidate for assay optimization, as successful miR-223 amplification and quantification had been achieved by previous thesis students (Nilsson, 2021; Marinkovic, 2021), where the same methods as used in this study were employed. The results obtained during the optimization phase with miR-223 could thus be compared to those obtained by previous thesis students, to determine whether potential issues observed in this study might be due to assay design or the reagents used. Two-tailed RT-qPCR on synthetic, as well as circulating miR-seps 6 could then ensue. The amplification efficiency of the two-tailed RT-qPCR assay should then be assessed through constructing a linear standard curve using synthetic miR-seps 6. Lastly, it should be discerned whether detection and amplification of circulating miR-seps 6 is possible by attempting to amplify circulating miR-seps 6 templates present in non-spiked RNA extracts, followed by absolute quantification using the synthetic miR-seps 6 linear standard curve.

Materials and methods

Procuring of and storage of human blood plasma

For sample collection, 24 mL of venous whole blood was extracted intravenously by a nurse at the university of Skövde and added to lavender top tubes containing EDTA (Grenier Bio-one). The tubes containing venous whole blood was tilted four times and placed on ice, and centrifuged at 2000 rcf (G) for 15 minutes at a temperature of 4°C, using the ScanSpeed 1580R (LaboGene). The separated plasma layer was then extracted and placed in a 15 mL falcon tube. The plasma was then transferred to 1.5 mL Eppendorf tubes into 500 µL and 300 µL aliquots. The plasma was stored at -80 °C to be utilized for downstream applications.

RNA extraction

Small RNA was extracted according to the miRNeasy serum/plasma advanced kit (Qiagen) protocol. A starting plasma volume of 100 µL was used as opposed to 200 µL, and as such, buffers RPL and RPP were downscaled accordingly. Buffer RPL was downscaled from 60 µL to 30 µL. Buffer RPP was downscaled from 20 µL to 10 µL. During manual extraction, 105 µL of supernatant arising from centrifugation was aspirated, and combined with 105 µL isopropanol (Fisher scientific), after which 200 µL of the combination was then aspirated and submitted to further procedures as per the miRNeasy serum/plasma advanced kit protocol. Where plasma samples were spiked, a designated copy number (10^5, 10^6, or 10^7 copies) of synthetic miR-seps 6 (Integrated DNA technologies) was inoculated into the extraction after step 3 of the miRNeasy serum/plasma advanced protocol. RNA extraction was also performed semi-automatically, using a QIAcube® (Qiagen). Steps 1 to 6 of the miRNeasy serum/plasma advanced kit were followed, after which 100µL of supernatant was placed in the QIAcube® for processing according to the QIAcube® miRNeasy serum/plasma advanced (Qiagen) protocol. Where samples were spiked, a designated copy number (10^5, 10^6, or 10^7 copies) of synthetic miR-seps 6 miRNA (Integrated DNA technologies) was inoculated into the extraction after step 3 of the miRNeasy serum/plasma advanced protocol.

Quantity and purity analysis

A total of 28 non-spiked sample eluates were analysed, of which 16 were extracted manually, and were denoted as non-spiked manual (NSM). Semi-automatically extracted samples (n=12), where the QIAcube® was used for extraction, were denoted as non-spiked automated (NSA).

After the RNA extractions were completed, eluates were placed on ice until the quantity of the samples were measured using Qubit microRNA assay kit (Thermo Fisher) along with the Qubit 4.0 fluorometer (Thermo Fisher), where 2 µL of each eluate was used. For the quantity analysis, 11 samples were measured in triplicate, and 5 were measured in duplicate, using the Qubit 4.0 (Qiagen). The averages where then calculated and used for analysis (Appendix 1).

The purity was measured using a nanodrop 2000 (Thermo Fisher), where 1 µL of each eluate was used. For the purity analysis, 4 NSM samples were measured in triplicate, using a nanodrop 2000 (Thermo Fisher) and their averages calculated (Appendix 1), which was used during analysis. After the quantity and purity analyses were completed, the eluates were stored at -20°C.

Two-tailed RT-qPCR

MiR-seps 6 was converted to cDNA and quantified by using the two-tailed RT-qPCR protocol (TATAA biocentre). Conversion to cDNA was done with Grandscript cDNA FreePrime kit (TATAA biocentre), which was downscaled from 20 µL reaction volume to 10 µL. The reagents volumes as per the Grandscript cDNA FreePrime kit user manual (TATAA biocentre) were thus appropriately adjusted and applied. Each reaction volume contained 50 nM two tailed primer (Integrated DNA technologies) designed to target miR-seps 6. A sample volume of 4 µL was used for each non-spiked eluate, as well as no reverse transcriptase (-RT) controls, and positive controls consisting of manual and semi-automated spiked 10^5 copies synthetic miR-seps 6 sample eluates. To create a standard curve, 1 µL of 10^{12} copies synthetic miR-seps 6 was reverse transcribed and was serially diluted after cDNA synthesis. Each reaction volume also contained 0.2 µL of Rox (BioRad), resulting in a final concentration of 500 nM. For the PCR (Polymerase chain reaction), cycling conditions as described in the Grandscript cDNA FreePrime kit user manual (TATAA biocentre) was applied. After adding all components apart from the GrandScript FreePrime Reaction Mix (5x) (TATAA biocentre) and the GrandScript RT Enzyme (TATAA biocentre), the reactions were incubated at 65°C for 5 minutes, after which the GrandScript FreePrime Reaction Mix (5x) and the GrandScript RT Enzyme were added. The reactions were then incubated at 42°C for 45 minutes followed by 85°C for 5 minutes using a PTC-200 Gradient Thermal Cycler (MJ Research). After the thermocycling was complete, the cDNA reactions were placed on ice, and submitted to downstream applications.

Quantification of the cDNA was done by using SYBR grandmaster mix (TATAA biocentre), which was downscaled to a 10 µL reaction volume. Each reaction contained 200 nM forward and reverse primers (integrated DNA technologies) and 5 µL TATAA SYBR® GrandMaster® Mix (2x). A template volume of 2 µL cDNA, arising from non-spiked manual and non-spiked semi-automated eluates were used as template and was done in triplicate. Spiked manual and spiked semi-automated (10^5 copies synthetic miR-seps 6) served as positive control. For cDNA sample arising from eluates, a corresponding -RT (no reverse transcription enzyme control) was prepared, containing the same template. NTC (no-template control) was prepared by adding nuclease free (NF) water (Merck) instead of template. For the linear standard curve, 1 µL of each miR-seps 6 cDNA serial dilution was used as template, ranging from 10^{10} copies/µL to 10^2 copies/µL and was done in triplicate.

An AriaMx Real-time PCR System (Aligent) was used to perform the qPCR reactions. The thermal profile used consisted of three segments, namely, hot start, amplification, and melt. For hot start, 95°C was held for 30 seconds, after which the amplification segment then ensured, which consisted of three steps, where 95°C was held for five seconds, followed by 15 seconds at 60°C and finally for 30 seconds at 72°C, at which point amplification measurements were taken. The amplification segment ran for 40 cycles. The melt segment consisted of 30 seconds at 95°C

followed by 30 seconds at 65°C and finally 30 seconds at 95°C. Measurements taken during the melt segment occurred where the temperature increased from 65°C to 95°C.

Optimization

For optimization, all extraction procedures and quality control analysis were done as described above. Human blood plasma which was extracted in 2021 and stored at -80°C was used for the optimization phase. To determine whether the two-tailed RT-qPCR is optimised with regards to reagent concentrations and cycling conditions, a linear standard curve using a 10-fold serial dilution series of synthetic miR-223 (integrated DNA technologies) ranging from 10^{11} copies to 10^{3} copies was created. It was also attempted to quantify circulating miR-223 as well as synthetic miR-223 that were spiked in during step three of the miRNeasy serum/plasma advanced protocol (Qiagen). The two-tailed primers, forward and reversed primers were sourced from integrated DNA technologies. All conditions with regards to reagent concentrations, reaction volumes and cycling conditions were identical to those used on miR-seps 6, as described above.

Inter-plate calibrator

As two qPCR runs were done an inter-plate calibrator was required to utilize the results originating from different qPCR runs in parallel. One concentration (10^{6} copies synthetic miR-seps 6) was used as an inter-plate calibrator, where the data arising from the second qPCR run normalized against the Cq value obtained from 10^{6} copies in the first run. A benchmark fluorescence (ΔR) of 50 was chosen for the first run, yielding a Cq value of 23.21 for 10^{6} copies synthetic miR-seps 6. During the second run, the threshold fluorescence (ΔR) was adjusted to 43, resulting in the same Cq value (23.21) for the 10^{6} copies synthetic miR-seps 6.

Absolute quantification

To absolutely quantify the circulating miR-seps 6 presents in eluates of non-spiked samples, the linear standard curve which was constructed from miRseps-6 cDNA was used. The following equation was used: y=mx + b. As the linear standard curve can serve as a constant in this project, a more specific equation would be $log\ copies = \frac{Cq-b}{m}$. To account for the volumes used, the value obtained was then divided by 4, resulting in log copy number/µL of cDNA reaction

To determine the efficiency of the linear standard curve, the equation $E = \frac{-1}{m}$ was used. To determine the % efficiency, $\%E = (E - 1) \times 100$ was used.

Data analysis

SPSS 27 for windows (IBM) was used for all statistical analysis. A significance level of $p \leq 0.05$ was used. In all statistical analyses, the Shapiro-Wilk test was used to determine normality, and the appropriate statistical test applied based on the hypothesis tested. To determine whether there was a significant difference between the distributions of concentrations with regards to manual and semi-automated means of extraction, a Mann-Whitney U test was employed. Similarly, a Mann-Whitney U test was also used to determine whether there is a significant difference between manual and semi-automated means of extraction regarding the distribution of A260/A280 ratios. An independent samples T-test was used to find whether there is a significant difference between manual and semi-automated means of extraction with regard to the miR-seps 6 log copy number/µL cDNA, as determined through absolute quantification. Finally, Pearson´s correlation was used to determine whether a relationship exists between A260/A280 ratios and miR-seps 6 log copy number/µL cDNA, as well as whether a relationship exists between RNA concentration and miR-seps 6 log copy number/ µL cDNA.

To create a linear standard curve, along with calculations concerning the linear standard curve, Microsoft Excel for Microsoft 365 (2020) was used.

Results

Comparison between manual and semi-automated extraction

To compare the two means of extraction, namely manual and semi-automated extraction, both quantity and purity analysis was done.

Of the NSM samples (n=16), 6 yielded small RNA concentration results that were too low to measure, which were corrected using the LOQ/$\sqrt{2}$ equation (Youssef, 2021; Succop et al., 2004) resulting in replacing the unquantifiable result with 0.177 ng/µL. All NSA samples (n=12) yielded quantifiable results. The median concentration for NSM samples (n=16) were 0.43 ng/µL (IQR= 0,36) and the median concentration for NSA samples (n=12) were 0.42 ng/µL (IQR=0,15). To test for normality, a Shapiro-Wilk test was used, which indicated a non-normal distribution, followed by a Mann-Whitney U test, which indicated no significant difference in the distributions of concentrations between NSM and NSA samples (Figure 1A).

NSM samples (n=16) delivered an A260/A280 ratio median of 1,87 (IQR= 0.54) and NSA samples (n=12) yielded an A260/A280 ratio median of 0.99 (IQR= 0.24). A Shapiro-wilk test was employed, indicating non-normal distribution, followed by a Mann-Whitney U test, which indicated that there is a significant difference between NSA and NSM samples with regards to the distribution of A260/A280 ratios (p= 0.00) (Figure 1B).

A) B)

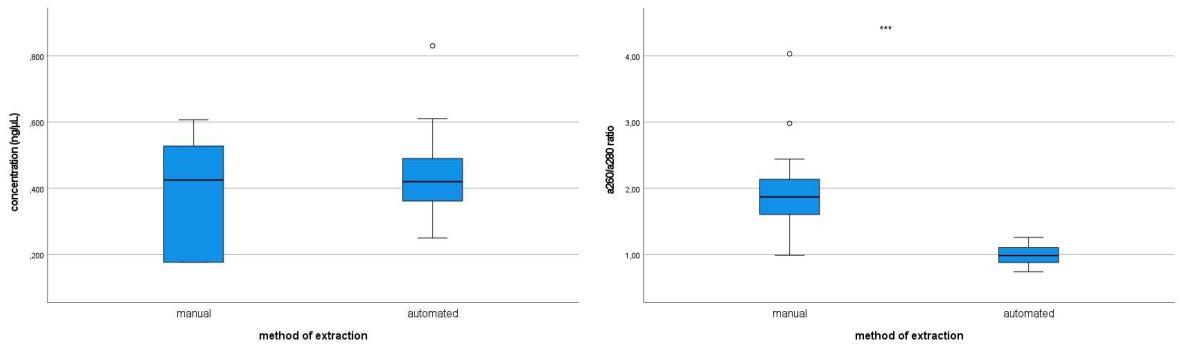


Figure 1: A) Box plot representing the interquartile range and error bars (CI: 95%) of the small RNA concentrations that resulted from manual extraction (n=16) and semi-automated extraction (n=12), with an outlier represented by a circle. B) Box plot showing the interquartile range, error bars (CI: 95%) and medians of the A260/A280 ratios derived from manual extraction (n=16) and semi-automated extraction (n=12), and outliers represented by circles. The triple asterisks denote significance of < 0.001, which was determined by a Mann-Whitney U test.

Quantitative polymerase chain reaction

A linear standard curve was constructed using a 10-fold serially diluted miR-223 series (Figure 2). The resulting slope was -4.042, which corresponds to a % efficiency of 76.76%, and the determinants of coefficient (R^2) was 0.998. Copy numbers 10^3 and 10^4 were highly variable between replicates in terms of Cq value (data not shown), and as such were excluded from the linear standard curve. None of the non-spiked nor the samples spiked with synthetic miR-223 reached the threshold fluorescence (data not shown).

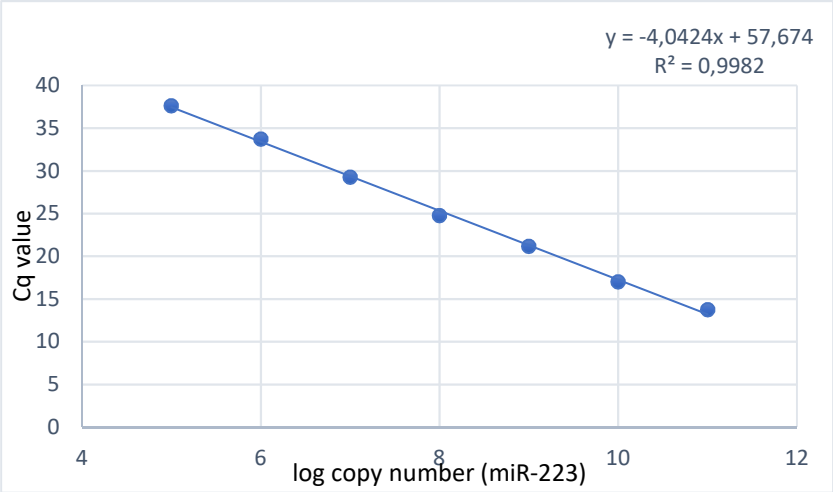
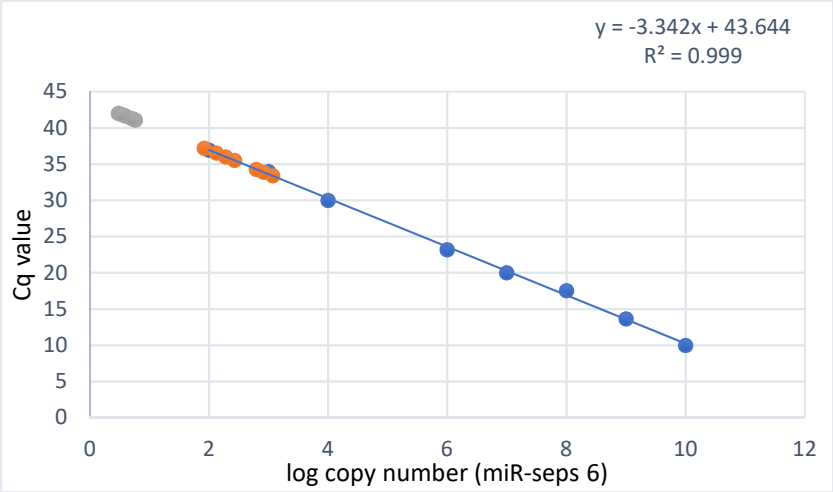


Figure 2: A linear standard curve constructed by using synthetic miR-223. The log copy number of miR-223 is present on the x-axis and the Cq values are present on the y-axis. The linear standard curve equation is y=-4.024 + 56.674 and the R^2 is 0.998.

Within the miR-seps 6 serial dilution series (Appendix 2), each of the copy numbers, 10^{10}; 10^8; 10^7; 10^6; 10^4; 10^2 contained a replicate, which significantly deviated in Cq value from the remaining replicates. The outliers were discarded, resulting in duplicates as opposed to triplicates. The concentration 10^5 contained highly variable Cq values (Appendix 2) and was thus discarded from the miR-seps 6 linear standard curve (Figure 2A). The linear standard curve containing synthetic miR-seps 6 was consulted to determine reaction efficiency (Figure 3A). The slope of the standard curve was -3.342, which corresponds to an %E of 99.16%. The coefficient of determinant was also observed (R^2= 0.999).

A)



B)

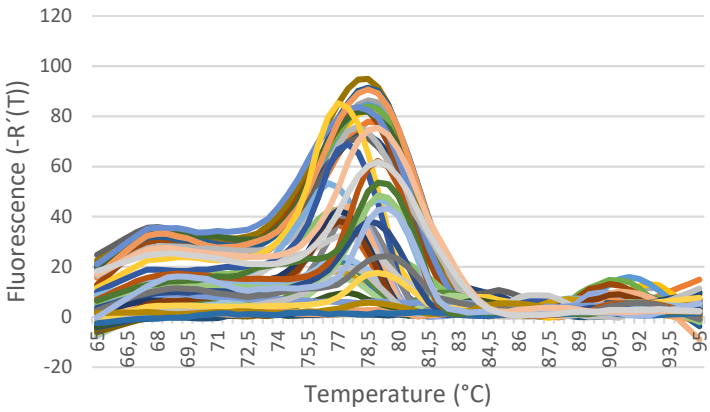


Figure 3: A) A linear standard curve with the equation y= -3.342x + 43.644. The average Cq value of each synthetic miR-seps 6 replicate (y-axis) was calculated and plotted against its log copy number (x-axis). The R^2 value was 0.999. The orange dots represent the quantified values of NSM (n=4) and NSA (n=3) samples.The grey dots are representative of the quantifiable NSM (n=4) and NSA (n=3) samples (n=7) in terms of log miR-seps 6 copies/µL of cDNA reaction. B) Melting curves of synthetic miR-seps 6 cDNA templates, as well as circulating miR-seps 6 cDNA, where the melting temperature is present on the x-axis and the fluorescence (-R'(T)) is present on the y-axis.

Of the NSM (n=6), and NSA (n=6) samples, where circulating miR-seps 6 were targeted through two-tailed RT-qPCR (n=12), 7 surpassed the benchmark fluorescence (ΔR) (Table 1), of which 3 were NSA and 4 were NSM. The Cq values were absolutely quantified by converting them to log copy number through using the linear regression equation, along with the linear standard curve (Figure 3A). The absolutely quantified values of both NSA and NSM samples are represented by orange dots (Figure 3A). The grey dots (Figure 3A) represent absolutely quantified values, transformed to log copies miR-seps 6/µL cDNA (Table 1), where the absolutely quantified values, as shown by the orange dots were divided by 4, as to account for the volume of cDNA template used.

The melting curves of synthetic miR-seps 6 and circulating miR-seps 6 cDNA templates (Figure 3B) indicate well-defined peaks between 79 °C and 81 °C. A slight secondary peak can be observed in wells containing higher concentrations of synthetic-miR-seps 6 cDNA.

Table 1: non-spiked manual (NSM) and non-spiked semi-automated (NSA) extractions, with their Cq values, miR-seps 6 copies/µL cDNA and standard deviations from the mean.

SAMPLE NAME	CQ VALUE	LOG-COPIES/µL CDNA	SD
NSM 4*	35.53	0.61	-
NSM 6	33.39	0.78	0.06
NSM 8	33.89	0.73	0.07
NSM 9	34.30	0.70	0.14
NSA 2	36.02	0.57	0.05
NSA 3	37.22	0.48	0.06
NSA 4	36.55	0.53	0.14

* NSM 4 only had one data-point, and as such, a standard deviation cannot be calculated

To determine whether there is a significant difference between the manual and semi-automatic method of extraction, with regard to miR-seps 6 log copies/µL cDNA specifically, the means of NSA (n=3) and NSM (n=4) samples (Table 1) were compared. Manual extraction yielded a mean log copies/µL cDNA value of 0.70 (SD= 0.07; 95% CI= 0.59 – 0.81). Semi-automated extraction yielded a mean log copies/µL cDNA value of 0.53 (SD= 0.05; 95% CI= 0.41 – 0.64) (Figure 4A). The Shapiro-Wilk test was consulted, which indicated normal distribution, followed by an independent samples t-test, which yielded a p-value of 0.01, indicating that there is a significant difference between the means of manual and semi-automated extractions.

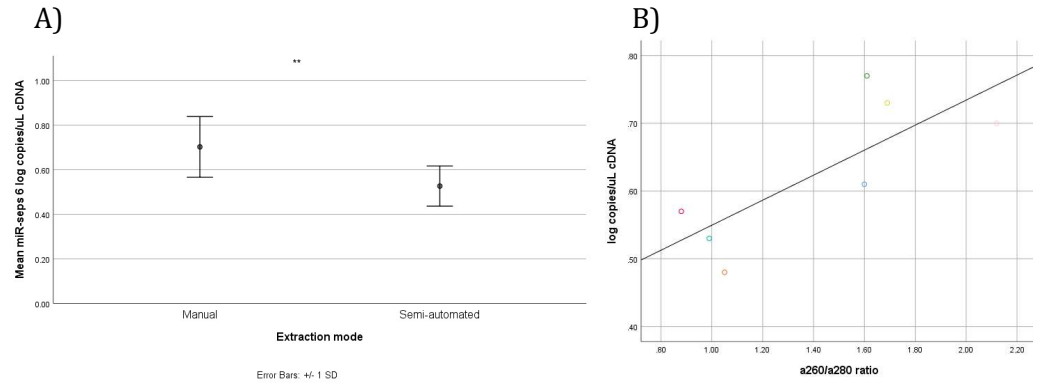


Figure 4: A) A comparison between NSM (n=4) and NSA (n=3) extraction miR-seps 6 log copies/µL cDNA reaction. The means are depicted by the dots present in the error bars. The error bars represent the standard deviation (SD±1) from the mean. An independent samples t-test was performed, which indicated a significant difference between manual and semi-automated extraction with regards to log copies/µL cDNA (p=0.01), which is denoted by the double asterisks (p= <0.05). B) A scatter plot of all quantified non-spiked extracts (n=7), where miR-seps 6 log copy number per µL cDNA reaction (y-axis) were plotted against A260/A280 ratios (x-axis). The circles within the plot represent individual samples of both NSM and NSA origin. The R^2 is 0.599. A Pearson's correlation was performed, which indicated a positive relationship exists between miR-seps 6 log copies/µL cDNA (r= 0.77; p=0.04).

To determine whether a relationship exists between A260/A280 ratios and log copy numbers/µL of cDNA reaction, a Pearson´s correlation was performed (Figure 4B), yielding a Pearson´s R-value of 0.77 (p=0.04), which indicated that a positive relationship exists between miR-seps 6 log copy number/µL eluate and eluate A260/A280 ratios.

Discussion

During this project, an analysis of the extraction and quantification of a potential sepsis miRNA biomarker was done in the hopes of creating a standardized protocol which yields repeatable quantification results. To determine whether results are repeatable, biological variance was eliminated by using plasma obtained from a single, self-determined to be healthy, individual. As the plasma samples were biologically identical, variance that was observed among samples might have been due to technical factors introduced through handling bias including the amount of blood withdrawn during phlebotomy and amount of starting plasma used due to pipetting inaccuracy. Careful consideration was applied to ensure that samples were handled in a like manner pertaining to RNA extraction and sample storage, as to limit the effects of technical variance. Plasma extracts were stored at -80 °C as to ensure the integrity of the miRNA present within the sample, by limiting degradation (McDonald et al., 2011).

To amplify miRNA´s, total RNA is required to be extracted, whilst removing contaminants that could act as inhibitors of downstream applications, in this case, RT and qPCR reactions. Plasma contains numerous contaminants that interfere with downstream applications that may hinder reverse transcription reaction efficiency or amplification efficiency, such as RNases and other proteins, (Wright et al., 2020). It is thus pivotal to optimize the extraction protocol with regards to the extraction kit used and starting volume of plasma, with the aim of decreasing inhibitor contamination, and increasing miRNA yield. The miRNeasy serum/plasma advanced kit (Qiagen) was determined to be the most suitable extraction kit based on RNA yield, as well as for the fact that it does not contain phenol, which is a contaminating substance that could act as an inhibitor during PCR (Nordén, 2020).

Quantity and purity analysis

During manual non-spiked extractions, many of the samples resulted in small RNA concentrations that were too low to measure with the Qubit 4.0 (Qiagen) (Appendix 1). To disregard the data points below the limit of detection (LOD) could result in a left censored distribution (Succop et al., 2004), leading to a higher estimation of the mean. RNA extractions that resulted in a "too low to measure" might still contain RNA, and as such concentrations below the LOD are valid data points, required to discern whether there is a difference between the extraction efficiencies of manual and semi-automated extraction. To account for values below the LOD, the Limit of quantification (LOQ)/$\sqrt{2}$ equation (Succop et al., 2004) was used. The aforementioned equation was previously utilized on qubit values pertaining to small RNA concentrations by Youssef (2021). The LOD of the qubit microRNA assay is 0.5 ng/µL per assay (*Qubit™ microRNA assay kits user guide*, 2022). Seeing that 2 µL of the eluate was used as input volume for each assay, the LOD in this case was 0.25 ng/µL, the eluates that were undetectable were thus replaced with the constant 0.177 ng/µL. When comparing manual and semi-automated extraction after applying the Limit of quantification (LOQ)/$\sqrt{2}$ equation, it was found that the distribution of concentrations does not differ significantly ($p = 0.599$) (Figure 1A). A possible method to increase the sensitivity of the Qubit 4.0 (Thermo Fisher) is to increase the sample input volume. By increasing the input volume from 2 µL to 4 µL, the LOD would decrease from 0.25 ng/µL to 0.125 ng/µL (*Qubit™ microRNA assay kits user guide*, 2022), which is below the constant of 0.177ng/µL, as determined through the (LOQ)/$\sqrt{2}$ equation. It would have thus been beneficial to optimize the assay with regard to the input volume, to decrease to number of samples that had small RNA concentration below the LOD.

The median small RNA concentration of manual extractions is highly similar to that of semi-automated extractions (Figure 1A). The observed concentration falls in range concentrations observed by Youssef (2021), who similarly used miRNeasy serum/plasma advanced kit (Qiagen)

and the QIAcube® (Qiagen), which was 0,10 ng/µL (SD±0.19) for manual extraction and 0.48 ng/µL (SD±0.49) for semi-automated extraction. The observed extraction concentrations, however, differ from the concentrations observed by Lindeberg (2020) which was 0.69 ng/µL (SD±0.09) for manual extraction and 1.79 ng/µL (SD±0.69) for semi-automated extraction. A possible reason for this might be biological variance, as the blood plasma donors are different. Sharma et al. (2022) compared the extraction efficiencies of RNA from peripheral blood mononuclear cells in terms of manual extraction and semi-automated extraction using the QIAcube® (Qiagen), where it was found that there was no significant difference in the distribution concentrations (p=0.09) between the two means of extraction, which corresponds with what was observed in this study. Youssef (2021), however, found that there was a significant difference between manual and semi-automated means of extraction with regards to small RNA concentration (p=< 0.00), with semi-automated extraction yielding a larger small RNA quantity. During analysis, Youssef (2021) included spiked samples, whereas this study only uses non-spiked samples for comparison between manual and semi-automated extraction, as to limit technical variance. An advantage to using semi-automated means of extraction can be seen in the difference in IQR (Figure 1A), where semi-automated extraction yielded a lower IQR (0.15) than manual extraction (0.36). A lower IQR indicates that the result is more repeatable, which is advantageous when creating a standard detection method.

With regards to purity, a significant difference between manual and semi-automated extraction with regards to the distribution of A260/A280 ratios (p = 0.00) was observed (Figure 1B). The analysis indicated that manual extraction yielded a higher A260/A280 ratio (1.87; IQR:0.54) as compared to semi-automated extraction (0.99; IQR:0.24) (Figure 1B). An A260/A280 ratio of between 1.8 and 2.0 indicated high purity (Lee et al., 2012), which was indeed achieved by manual extraction (Figure 1A). Sharma et al. (2022) did not observe a significant difference between manual and semi-automated means of extraction with regards to A260/A280 ratio (p=0.27). The variables used by Sharma et al. (2022), however, are different from the ones used in this study, with regards to the kits used, the type of tissue RNA was extracted from and the type of RNA that was quantified. Sharma et al. (2022) also compared the mean concentration, as opposed to the distribution of concentrations. It was elected to determine whether there is a difference between manual and semi-automated means of extraction with regard to the distribution of data. This is because the question of reproducibility is fundamental in determining whether a specific assay can be relied upon during clinical use. As the risk for technical variance, and consequently, an increase in the distribution of data has the potential increase the risk for that false-positives or false-negatives results occurring.

Of note with regards to the quantity and purity analysis, using the Qubit microRNA assay kit (Thermo Fisher) along with the Qubit 4.0 fluorometer (Thermo Fisher) and the Nanodrop 2000 spectrophotometer (Thermo Fisher) respectively, is that neither of these methods can measure miRNAs exclusively (Garcia-Elias et al., 2017). The Qubit microRNA assay (Thermo Fisher) along with the Qubit 4.0 (Thermo Fisher) measures small RNA species, which includes miRNAs, and the Nanodrop 2000 (Thermo Fisher) measures absorbance, which cannot discern between miRNAs, other RNA species and DNA, as all the aforementioned molecule types absorb at a wavelength of 260nm (Garcia-Elias et al., 2017). Wright et al. (2020) and Gracia-Elias et al. (2017) concur that the Qubit microRNA assay provides a better platform for estimating miRNA content compared to spectrophotometric measurements by means of a Nanodrop, due to its consistency and its ability to specifically quantify small RNA species, whilst decreasing the influence that contaminants such as larger RNA or DNA present in the sample might have on measurements. The Nanodrop 2000 (Thermo Fisher), regardless of its inability to measure miRNAs specifically, still provides a valuable insight into the presence of contaminating substances that absorb at 280 nm.

The difference in A260/A280 ratio between manual and semi-automated extraction (Figure 1B) could be due to various factors, two examples of which, that are variable between manual and semi-automated extraction, is the amount of isopropanol added, and the centrifuge speeds. Regarding the isopropanol ratio, the QIAcube® (Qiagen) follows a program specific to the miRNeasy serum/plasma advanced kit (Qiagen), which assumes a 200 µL serum/plasma starting volume. It thus assumes that 230 µL supernatant will be withdrawn and added to the machine for processing. The isopropanol ratio of 1 to 1 thus results in 230 µL isopropanol to be added. When 100 µL starting volume is used, and 100 µL supernatant is added to the machine for processing to be comparable to the manual extraction process, 230 µL isopropanol is added by the QIAcube®. This might affect extraction efficiency, as it has been shown that variations in isopropanol ratio during nucleic acid precipitation results in variable miRNA isolation efficiencies (Li et al., 2020). The QIAcube® has a maximum relative centrifugal force (RCF) of 12000 (QIAcube® user manual, 2018), which is less than the centrifuge used during manual extraction, which has a maximum RCF of 14100. To determine whether a difference in RCF was a source of variation, the small RNA extraction efficiencies arising from extractions where different RCF were applied during manual extraction should have been experimentally determined, which was not performed in this study.

Differences with regards to the distribution of concentrations and A260/A280 ratios within each method of extraction (Figure 1A) may be due to technical variance, as biological variance is eliminated by sourcing plasma from a single individual. The risk for technical variance is increased due to plasma being considered a challenging biofluid to work with, particularly when studying miRNA´s present in plasma, as a result of various factors, including their low copy number, and structural properties (Gevaert et al., 2018). As mentioned, a significant difference was observed between manual and semi-automated means of extraction with regards to the distribution of A260/A280 ratios ($p=0.00$) (Figure 1B). A possible reason for this is the reduction of the number of steps requiring manual input by using semi-automated means of extraction. Variation within the semi-automated method of extraction might be because of manual input required during the initial phases of the extraction protocol. A particular step, namely, incubation time with denaturation buffer has been shown to have a substantial effect on the variance regarding concentration of miRNA (McDonald et al., 2011). It was observed that during the addition of buffer RPP, which a white precipitate forms, consisting mainly of proteins (Qiagen, 2020). The precipitate tends to become more difficult to disperse with increasing incubation time. Ensuring that the precipitate is dispersed through vortexing is pivotal in achieving stage separation. If the precipitate is not adequately dispersed, the supernatant that results after centrifugation tends to be milky as opposed to clear, which indicates contamination with cellular components. It is recommended in the miRNeasy serum/plasma advanced kit handbook (Qiagen) that vortexing be done for 20 seconds. This, however, might not be adequate to disperse the precipitate. Visual inspection must thus be employed to check for any precipitate still present, and if present, the sample must be vortexed until said precipitate has dispersed. Technical variance might also be the result of pipetting inconsistencies with regards to starting volumes of plasma. The miRNeasy serum/plasma advanced kit comprises of a few steps where variability in volumes may be implemented, such as the starting amount of plasma, the amount of supernatant aspirated, and the elution volume. To instil consistency, these variables require to be standardized.

Certain substances such as phenol, salts and carbohydrates absorb at 230 nm (Wang et al., 2010). Most samples analysed had an A260/A230 ratio of below 1 (Appendix 1), which falls outside of the ratio (~2.0) that is typically considered pure (Lee et al., 2012). As the miRNeasy serum/plasma advanced kit does not contain phenol, it is likely that there is a substantial salt contamination, which may have arisen from the kit components, or the plasma sample. Of note is the small amount of small RNA present in plasma (Mlcochova et al., 2014), which may result in the distortion of the A260/A230, because of the minuscule amount of small RNA present in the

resulting eluate, along with a standard number of substances that absorb at 230nm, thus giving the impression that the sample is contaminated (El Khoury et al., 2016).

Two-tailed RT-qPCR

Optimization

To create an effective standard method of detecting biomarker miRNA, which is relevant to this sepsis project, a continuation of methods used in previous thesis work was employed, as to eliminate variation that might skew results, leading to data which is incompatible with previous research within the project. As such, parameters used by previous students (Youssef, 2021; Nilsson, 2021) with regards to reaction volumes, reagent concentrations, as well as eluate and cDNA volumes were used. To test whether the reaction conditions were effective, a linear standard curve was created using synthetic miR-223 (Integrated DNA technologies) (Figure 2). The % efficiency (%E= 76.76%) was suboptimal, as a minimum % efficiency for a linear standard curve to be deemed acceptable is 90% (Taylor et al., 2010). The R^2 of 0.998 indicates that there is a linear relationship between the data points, which validates the efficiency (Taylor et al., 2010). According to the SYBR grandmaster mix protocol (TATAA biocentre), a reaction volume of 20 µL is prescribed. Similarly, the SYBR grandmaster mix protocol (TATAA biocentre) recommends a primer concentration of 1-2 pM, however, a concentration of 50 nM was used, as did Youssef (2020) and Nilsson (2021). The decrease in reaction volume and/or the increase in primer concentration might have been contributing factors pertaining to the sub-optimal reaction efficiency observed in the linear standard curve created with synthetic miR-223 (integrated DNA technologies) (Figure 2), however, the reaction efficiency observed in the linear standard curve created with synthetic miR-seps 6 was optimal (Figure 3A). Similarly, in creating a linear standard curve with synthetic miR-223, using the same reaction conditions, Nilsson (2021) obtained a % efficiency of 103% and Youssef (2021) obtained a % efficiency of 100%, using synthetic miR-210. Other factors, such as the handling of the synthetic miR-223 templates regarding freezing and thawing, and sample storage might be contributing factors.

miR-seps 6

The synthetic miR-seps 6 linear standard curve (Figure 3A) conforms with what is indicative of an optimal assay design (Taylor et al., 2010), where efficiency refers to how effective the assay is at converting reaction components to a fluorescence signal, which is then detected, and the R^2, which indicates how well the data points conform with the linear standard curve (Taylor et al., 2010). Synthetic miRNA, however, may differ from circulating miRNA in terms of physiochemical properties (Redshaw et al., 2013), and as such, may not be optimal in determining reaction efficiency. Schamberger and Orbán (2014) proposes using extracted miRNA, which is then serially diluted in smaller steps, such as 1.5-fold dilutions as opposed to 10-fold dilutions, to create a linear standard curve. Another source of variation might be differences between the samples and standards in terms of reaction contents. It has been shown that background RNA influences RT reaction efficiency (Miranda & Steward, 2017). During this study, the RT reaction efficiency alone could not be determined, as the linear standard curve was created based on the qPCR data, which considers the efficiencies from both enzymatic reactions, namely the PCR and the RT reaction jointly, as to determine the reaction efficiency of the assay as a whole. Due to variation observed among triplicates within the synthetic miR-seps 6 cDNA linear standard curve, certain technical replicates were removed where significant deviation from the other two, due to handling bias, was observed (Appendix 2). Where there was no conformity with regards to Cq within a triplicate, the entire triplicate was removed. Dijkstra et al. (2011) proposes that if a technical replicate presents as an outlier because of technical error, where the Cq deviates by more than 0.5 cycles,

that the data point should be removed, as to ensure valid data. De Ronde et al. (2017) however, reasons that if the 0.5 Cq deviation is applied to the entire range of Cq values, that valid data points will be excluded, and as such, the Poisson distribution should be applied. The Poisson distribution considers efficiency along with Cq value and determines the deviation among replicates that should be considered valid (De Ronde et al., 2017). During this study, Poisson distribution was applied when discerning which data points within replicates should be excluded. An exemption was made regarding the triplicate containing 10^6 copies synthetic miR-seps 6 cDNA, was made, due to it being used as an inter-plate calibrator. The difference in Cq was 0.55. Poisson distribution deemed a Cq difference of 0.5 to be appropriate.

When observing the melting curves derived from synthetic miR-seps 6, as well as circulating miR-seps 6, well defined peaks between 79 and 81 °C were present (Figure 3B), indicating good specificity of the RT, forward, and reverse primers (Taylor et al., 2010). A slight second peak can be seen in the inter-plate calibrator as well as in wells containing higher concentrations of synthetic miR-seps 6 at higher concentrations, as well as two NTC wells. Unequal disassociation due to a region rich in CG content, resulting an uneven disassociation of the amplicon may be the reason for the slight secondary peak (Robinson et al., 2006). The likelihood of primer-dimers forming is mitigated by the observation that in wells containing a lower concentration of template, a secondary peak is absent. Where a lower cDNA template concentration is present, a higher concentration of primer will be present, which will increase the chances for primer dimers due to less competition with regards to forming dsDNA. The risk of primer dimers influencing the observed fluorescence remains plausible, as SYBR green is an intercalating dye (Ye et al., 2019), and as such can intercalate within bonds formed between two primers and as such may affect the fluorescence detected. As no amplification was observed in -RT controls, it can be deduced that secondary peaks are not a result of gDNA contamination.

Of the 12 non-spiked samples that were subjected to two-tailed RT-qPCR, 7 showed quantifiable amplification results. Of the quantifiable results, 4/7 originated from manually extracted samples, and 3/7 resulted from semi-automatically extracted samples. As the number of samples that were subjected to two-tailed qPCR were too low to draw a conclusion on whether manually extracted samples yielded superior results, further analysis is recommended. As amplification was seen, it can be concluded that the assay can detect and amplify miR-seps 6. All the quantifiable non-spiked eluates produced a Cq value between 33.39 and 37.22 (Table 1). To enable quantification of miR-seps 6 copies, absolute quantification ensued, utilizing the synthetic miR-seps 6 linear standard curve (Figure 3A). Upon observing the differences in standard deviation between manual extraction and semi-automated extraction (Figure 4A), semi-automated extraction delivered a smaller standard deviation (SD: 0.05), as compared to manually extracted non-spiked samples (SD: 0.07) with regards to miR-seps 6 log copies cDNA/µL, which indicates better consistency. If a certain diseased state results in a down-regulation of miR-seps 6, a risk exists for the Cq values to be above the LOD, which will render the results inconclusive. As the observed Cq values were relatively high (Figure 3A; Table 1), a strategy should be implemented to decrease the Cq values to increase accuracy and decrease the risk of surpassing the LOD of the assay. An increase in qPCR reaction volume from 10 µL to 20 µL might be advantageous, as a larger cDNA template volume can then be added, which will increase the amount of template present in the qPCR reaction volume, thus reaching the Cq after fewer cycles. As larger volumes may then be used, more consistent and accurate results may also be expected. It has been shown that total RNA integrity is severely affected by freezing, and as a result, have a detrimental effect on determining expression levels of miRNA using qPCR (Ibberson et al., 2009). Taking this into account, it could be beneficial to extract and measure miRNA expression directly after sample procurement as standard protocol to increase miRNA yield.

Various factors might increase the risk of false-positive results with regards to Cq values. These include gDNA contamination, haemolysis, and RT primer tendency to anneal to pre or pri-miRNA. To account for this, Androvic et al. (2017) developed a RT primer that contains a stem-loop, which results in the primer favouring RNA/DNA duplex formation with ssRNA and thus avoiding annealing to gDNA, or to pri and pre-miRNA, as these species contain a stem-loop (Pratt & MacRae, 2009). Pre and pri-miRNA are also sensitive to RNase activity. As circulating miRNA is protected from RNase degradation through either AGO 2 association or encapsulation within exosomes, the presence of RNase would not affect circulating miRNA whilst breaking down immature miRNA. Haemolysis have been shown to increase the concentration of miRNA is present in erythrocytes (McDonald et al., 2011), however, it is not known whether this would influence miR-seps 6 concentrations, as it is not known whether miR-seps 6 is present in erythrocytes. Nordén (2020) observed no indication of haemolysis in eluates arising from miRNeasy serum/plasma advanced kit, indicating that the use of the miRNeasy serum/plasma kit might not be prone to causing haemolysis. During biobanking in the SepsIT® project, special care is taken with regards to sample handling, where plasma samples would only be thawed once, and would immediately be subjected to RNA extraction as to decrease the risk of hemolysis occurring (A.K Pernestig, personal communications, 19/05/2022).

To determine whether a relationship between the log copy number miR-seps 6/µL cDNA and eluate small RNA concentration and/or A260/A280 ratios exist, a Pearson´s correlation was performed (Figure 4B). It was found that a positive relationship exists ($p=0.04$), which indicates that with increasing A260/A280, an increase in miR-seps 6 log copy number/µL cDNA was observed. It can thus be assumed that consistency with regards to A260/A280 ratios are required to ensure accurate quantification results, as A260/A280 ratios may affect miR-seps 6 yield. A Pearson´s correlation between RNA concentration and log copy numbers/µL eluate was also performed, which showed no significant correlation (data not shown). As the sample size was relatively small ($n=7$), a conclusion cannot be drawn, as it is typically suggested that a sample size of at least 25 is required to have sufficient confidence in Pearson´s correlation (David, 1938), however, the data suggests that further research should be done to determine whether A260/A280 ratios has a positive relationship with miR-seps 6 yield. Semi-automated extraction delivered a more consistent RNA A260/A280 ratio (IQR:0.24) (Figure 1B), as compared to manual extraction (IQR: 0.54) which consequently may result in more consistent Cq values. As consistency is required during diagnostics as to decrease the risk of false positives or false negatives, it seems appropriate to automate the extraction process as standard protocol, as the results seem to indicate that increased handling results in increased variance.

Limitations of this study

To improve the quality of this study, certain factors that might have been a source of variation could have been investigated experimentally. As a difference between manual and semi-automatic extraction was observed with regards to A260/A280 ratio, it would have been beneficial determine whether elution at 12000 RCF as opposed to 14100 RCF had a significant effect on A260/A280 ratio. Elution at 12000 RCF during manual extraction could have been performed and the A260/A280 ratio compared to the A260/A280 ratio arising from elution performed at 14100 RCF during manual extraction. Another limitation in this study was that only frozen plasma was used, which is inconsistent with practices that would be applied in a clinical setting. As the diagnosing of sepsis is time dependent, the plasma would be immediately subjected to diagnostic tests after procurement. It has been shown that freezing and thawing of plasma affects sample integrity (Ibberson et al., 2009).

As plasma samples originating from one individual had been used during this study, there is a risk that the results observed might not be comparable to plasma samples sourced from other individuals. This is an important aspect, as biological variance may result in differential expression of circulating miRNA, dependent on variables such as age, gender, and ethnicity (Kolhe et al., 2017; Noren Hooten et al., 2010; Pollard et al., 2018). Target isolation efficiency may also be affected by unique protein matrix profiles, which varies between individuals (Sanders et al., 2014). It would thus have been beneficial to use a larger sample size, that is representative of the general population to ensure repeatability. The subject in this study was also self-determined to be healthy, and no subjects diagnosed with sepsis were used in this study. It could thus not be determined whether the methods used in this study would lead to reproduceable results within a cohort diagnosed with sepsis.

Ethics and Prospects

The blood used in this project is to be donated from thesis students participating in the project. The students were informed and asked for consent. If a student was unable to donate blood, alternative arrangements could be made. The experiments were done on each student´s own blood samples, and as such, no additional consent was required. The experiments planned did not cause harm in any way, and the results will not pose ethically concerning ramifications.

In association with the multimarker panel, which includes pathogen detection, a decrease in unnecessary antibiotic administration may occur, thus decreasing the risk of developing antibiotic-resistant bacteria (A.K Pernestig, personal communications, 19/05/2022). The ability to accurately quantify miRNA´s will allow for new prognostic, as well as diagnostic biomarkers being discovered and effectively utilized for a variety of diseases and syndromes. MiRNA´s may be included in multivariate diagnostic assays, thus improving the sensitivity and specificity of existing diagnostic tests. The capability to analyse miRNAs can also be used to gain a better understanding of how miRNAs regulate gene expression through interaction with the transcriptome, leading to a better overall understanding of gene expression, which will allow for the advancement of personalised medicine.

Kolhe et al. (2017) observed a difference between biological male and biological female subjects with regards to miRNA content present in extracellular vesicles pertaining to individuals diagnosed with osteoarthritis. As a difference in miRNA expression between biological males and biological females may present in a diseased state, it is evident that biologically male and female subjects should be equally represented in studies aimed at validating miR-seps 6 as a sepsis biomarker, as to determine whether miR-seps 6 provides equal diagnostic potential regardless of gender.

Conclusion

During this study, three questions were investigated, namely, whether semi-automated RNA extraction from plasma yielded superior results compared to manual RNA extraction, whether the two means of extraction differed in terms of miR-seps 6 extraction efficiency, and whether miR-seps 6 was quantifiable using the two-tailed RT-qPCR method. The semi-automated means of extraction seemed to have yielded superior results in terms of consistency. No significant difference was observed with regards to RNA concentration, however, the distribution of A260/A280 ratios significantly differed, with semi-automated extraction being more consistent. As there is a positive correlation between Cq values and A260/A280 ratios ($p=0.04$), particular emphasis should be put on ensuring consistency of A260/A280 ratios, and as such, semi-automated extraction by means of the QIAcube® might be advisable.

The two-tailed RT-qPCR method has shown to be effective at amplifying circulating miR-seps 6 arising from 100 µL of human blood plasma. A linear standard curve, constructed from synthetic miR-seps 6 produced optimal amplification efficiencies, and the melt curve indicated a single product, which correlates with good specificity. It, however, is evident that optimization is required before clinical use of the assay, as the assay performed sub optimally on synthetic miR-223. This might be due to ineffective storage of primers, or contamination of the assay components with inhibitors. A problematic factor to consider with regards to the detection of circulating miR-seps 6 is the low copy number presents in human blood plasma. Downregulation of circulating miR-seps 6 might be a compounding effect with regards to the accuracy of the two-tailed RT-qPCR assay, and as such, methods should be implemented to increase the amount of template within the PCR reaction, such as increasing the PCR reaction volume. To determine whether the low copy number of miR-seps 6 observed is due to a low amount being present in plasma, or whether extraction efficiency is the cause, synthetic spike-ins should be utilized to determine extraction efficiency. Brunet-Vega et al. (2015) used six spike-in miRNA molecules typically not found in plasma to determine the extraction efficiency of the kit used. The extracted spike-ins were then subjected to RT-qPCR, yielding highly variable Cq values among extracts. During this project, spike-ins were utilized as a positive control, and too few data point (n=1) were available to effectively determine extraction efficiency.

As successful RNA extraction, using the miRNeasy serum/plasma advanced kit (Qiagen) and detection and amplification of miR-seps 6 using the two-tailed RT-qPCR method had been achieved during this study, the next phase of the project can continue. It will be attempted to extract, amplify and detect miR-seps 6 from plasma stored in a human biological material bank (biobank), where, if successful, may lead to the inclusion of miR-seps 6 in the multimarker panel, SepsIT®, which aims to aid in digital diagnostics of sepsis.

Acknowledgement

I would like to thank all the lecturers at the University of Skövde whom, through their expertise, provided me with the knowledge and experiences to reach this point in my academic journey. I would also like to acknowledge the critical role Anna-Karin Pernestig and Johan Nordén played in me performing this thesis project and thank them for their diligent and patient guidance throughout this project. Lastly, I would like to extend my gratitude to my fellow peers, whom I had the opportunity to work with over the past few years. It has truly been a fantastic experience and could not have done it without any of the elements listed above.

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Appendix 1

Table 1: Raw data derived from plasma extractions, where absorbance measurements and ratios, as well as concentrations are displayed.

SAMPLE	CONCENTRATION (NG/µL	A260	A260/A280	A260/A230
NSM 1	0.479	0,029 0,053 0,096	2,02	0,04
NSM 2	0.550	0.357 0.383 0.528	0,99	0,02
NSM 3	Too low	 0.081	1,025	0,44
NSM4	0.561	0.172 0.035 0.256	1,6	0,02
NSM 5	0.400	0.066	1,55	0,04
NSM 6	0.420	0.073	1,61	0,20
NSM 7	0.607	0.105	1,85	0,01
NSM 8	0.430	0.187	1,69	0,23
NSM 9	0.582	0.195	2,12	0,06
NSM 10	Too low	0.063	4,03	0,05
NSM 11	0,505	0.173	1,66	0,12
NSM 12	0.480	0.264	2,98	0,02
NSM 13	Too low	0,175	2,15	0,38
NSM 14	Too low	0,193	1,89	0,26
NSM 15	Too low	0,183	2,44	0,03
NSM 16	Too low	0,217	2,06	0,04
NSA 1	0.250	0.163	1.26	0.13
NSA 2	0.470	0.199	0.88	0.08
NSA 3	0.380	0.177	1.05	0.12

	0.350 0.400			
NSA 4	0.510	0.192	0.99	0.11
NSA 5	0.500 0.440 0.390	0.248	0.88	0.10
NSA 6	0.400	0.198	0.94	0.09
NSA 7	0.450 0.350 0.340	0.210	1.08	0.13
NSA 8	0.330	0.150	1.22	0.13
NSA 9	0.370 0.340 0.330 347	0.153	1.13	0.11
NSA 10	0.610	0.270	0.88	0.10
NSA 11	0.460 0.470 0.390 440	0.293	0.98	0.14
NSA 12	0.830	0.337	0.74	0.0
SM5 2	Too low	0,356	1,81	0,04
SM5 3	Too low	0,180	2,67	0,03
SM5 4	Too low	0,148	3,56	0,02
SM6 1	Too low	0,334	2,83	0,03
SM6 2	Too low	0,242	2,62	0,02
SM6 3	Too low	0,117	3,32	0,03
SM6 4	Too low	0,143	3,07	0,03
SM7 1	Too low	0,025	2,19	0,07
SM7 2	Too low	0,177	2,23	0,07
SM7 3	Too low	0,083	3,38	0,01
SM7 4	Too low	0,053	8,83	0,01
SA5 1	Too low	0,121	1,64	0,16
SA5 2	Too low	0,055	1,43	0.12
SA5 3	0,310	0,066	1,65	0,08
SA5 4	Too low	0,067	1,28	0,07
SA6 1	Too low	0,072	1,04	0,06
SA6 2	0,280	0,184	1,52	0,18
SA6 3	0,320	0,118	0,94	0,09
SA6 4	0,490	0,149	0,78	0,09
SA7 1	0,270	0,072	1,27	0,07
SA7 2	Too low	0,256	1,47	0,35
SA7 3	Too low	0,066	1,40	0,15
SA7 4	Too low	0,054	1,19	0,09

Appendix 2

Table 1) The well, replicate number, Cq and quantities of the miR-seps 6 10-fold dilution series used to create the linear standard curve.

Well	Replicate	Cq (ΔR)	Quantity (copies)
C7	10	36.95	2
C8	10	No Cq	No Cq
C9	10	36.95	2
C4	9	34.57	3
C5	9	34.3	1000
C6	9	33.02	1000
C1	8	32.12	4
C2	8	29.69	10000
C3	8	30.33	10000
B7	7	27.02	5
B8	7	26.3	100000
B9	7	28.26	100000
B6	6	22.93	6
B5	6	24.06	1000000
B4	6	23.48	1000000
B1	5	No Cq	7
B2	5	20.38	10000000
B3	5	19.7	10000000
A7	4	17.53	8
A8	4	17.59	100000000
A9	4	16.66	100000000
A4	3	13.73	9
A5	3	13.49	1000000000
A6	3	13.75	1000000000
A1	2	10.11	10
A2	2	11.66	10000000000
A3	2	9.88	10000000000

*Highlighted values have been discarded from the linear standard curve.