Master Degree Project

UNIVERSITY OF SKÖVDE

Assessment of manual and robotic miRNAs extraction methods with optimization of the two-tailed RT-qPCR technology for miRNAs detection as biomarkers from human plasma for early sepsis diagnosis.

Future diagnostics of sepsis

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Abstract

Sepsis is a life-threatening syndrome that occurs due to dysregulated body response to pathogenic infections. More than 30 million cases are recorded annually worldwide, with a high mortality rate of up to 40% of the recorded cases. Early diagnosis of sepsis will help clinicians to start proper therapy as early as possible and save lives. Circulating miRNAs in biofluids were found previously as potential biomarkers that can be used in a multi-marker panel to develop a rapid, friendly user diagnostic kit for the early sepsis diagnosis. This study assessed miRNAs from healthy donors' human plasma by two extraction methods, manually and robotically via QIAcube. In addition to optimizing two-tailed RT-qPCR (TATAA Biocenter) technology for miRNAs detection and quantification. The extraction of miRNAs was using miRNeasy® Serum/Plasma Advanced kit (Qiagen) for the two methods. Plasma was spiked in with synthetic miRNA-210 to ensure miRNA detection and was used as a positive control for the study. The concentration and the purity of the RNA eluates were measured and statistically analyzed to identify which method could be better in conventional laboratory practice. QIAcube results showed its ability to compete with manual RNA extraction protocols. However, more studies are required for RNA extractions with different kits using QIAcube. The two-tailed RT-qPCR technology successfully detected many miRNAs, but more samples are required to be tested for accurate conclusions. The results emphasize the ability of two-tailed RT-qPCR to detect and quantify miRNAs from human plasma as potential biomarkers in a multi-marker panel for early sepsis diagnosis.

Popular scientific summary

Sepsis is a syndrome that arises from a body fighting a bacterial, viral, or fungal infection. The body response causes dysregulation in many of the body functions. If the leading cause of sepsis is not detected as early as possible, the organs may shut down and end with organ failure and subsequent death. Unfortunately, the symptoms of a patient suffering from sepsis are very common to many other diseases, which makes it difficult to diagnose sepsis by clinical symptoms. Symptoms could be fever, chills, high heart rate, disorientation, pain or discomfort, and shortness of breath. Currently, blood culture is the gold standard to identify the microorganism that invades the body, causing an infection in organs or the bloodstream. However, this technique takes few days to give a result. During these few days, clinicians usually start general protocols once sepsis is suspected, like intravenous fluid replacement and supplemental oxygen. In addition, empirical broad-spectrum antibiotics are usually prescribed before the blood culture results are determined to prevent the patient from developing organ failure with severe hypotension named "septic shock." Meanwhile, unnecessary use of antibiotics may develop antibiotic resistance and weaken the immunity of the patient.

The demand of finding a method or a technique for the early diagnosis of sepsis is crucial. It has a considerable probability to save millions of lives from death or comorbidities. Researchers had found some biological components inside the human body that can be a sign of a specific disease or an infection. These components are named "Biomarkers", biomarkers can change their expression level either by elevation or down-regulation in the blood during a healthy state or an infection state. Available biomarkers for sepsis used up to date are C-reactive protein, lactic acid procalcitonin, and white blood cell elevation level. However, these biomarkers also change their expression level in inflammatory diseases. Thus, these biomarkers are not specific and may interfere with the diagnosis of several diseases. In Sweden, at the University of Skövde, researchers had been working on finding specific biomarkers such as proteins and nucleic acids that can help in the early diagnosis of bacterial sepsis. They aim to build a multi-marker panel that can combine several biomarkers along with vital parameters of the patient for early, rapid and accurate diagnosis of bacterial sepsis. One of the potential candidates as biomarkers in the multi-marker panel is microRNAs (miRNAs) from human blood. MiRNAs are short non-coding RNAs that play a vital role in regulating gene expression all over the body. It was also found that miRNA expression level changes with certain diseases like cancer and cardiovascular disorders. Recently, a multi-marker panel has been developed with potential miRNA candidates and needs to be validated. Nevertheless, the extraction and detection of miRNAs from human plasma are not optimized yet for the best practice and benchmarking, and that is the aim of this study.

There are plenty of researches that worked on different kits for miRNA precise extraction with high quality. Other studies focused on different techniques for miRNA detection and quantification. This study worked on extracting and detecting miRNAs from self-assessed healthy donor's human plasma. Meanwhile, two methods for extraction were used, manual and robotic by QIAcube machine. The quality and the purity of the RNA eluates including miRNA were assessed to know which method is better for optimum quality. Results showed that QIAcube could compete with the manual extraction method in miRNA extraction. The second part of the study was to optimize the two-tailed RT-qPCR technology from TATAA Biocenter to be able to detect and quantify naturally existing miRNAs from human plasma. Results were very promising; the technology showed its ability to detect and quantify miRNAs, and this technique can be used in the future to validate the developed multi-marker panel. Thus, the early detection of sepsis is one step closer to come true. The achievement will save lives, save medical costs, time, and healthcare workers' efforts to defeat ferocious syndrome like sepsis.

Table of Contents

Abbreviations

AGO	Argonaute proteins
AKI	Acute kidney injury
cDNA	Complementary Deoxyribonucleic acid
CI	Confidence interval
Cq	Quantification cycle
CRP	C-reactive protein
DGCR8	DiGeorge Critical Region 8 protein
IL-6	Interleukin-6
K2EDTA	Dipotassium ethylenediaminetetraacetic acid
miRNA	Micro Ribonucleic acid
MODS	Multiorgan dysfunction syndrome
mRNA	Messenger Ribonucleic acid
NGS	Next Generation Sequencing
NLCR	Neutrophil-lymphocyte count ratio
NRT	No reverse transcriptase
NS RNA	Non- spiked RNA
NS-S RNA	Non-spiked and spiked RNA
NTC	No template control
PCT	Procalcitonin
POC	Point of care
Pre-miRNA	Precursor microRNA
Pri-miRNA	Primary microRNA
QM	Qiagen manual extraction method
qPCR	Quantitative polymerase chain reaction
QQ	Qiagen QIAcube extraction method
RISC	RNA induced silencing complex
RNA	Ribonucleic acid
RT	Reverse transcription
RT-qPCR	Reverse transcription quantitative PCR
SIRS	Systemic inflammatory response syndrome
SOFA	Sequential organ failure assessment score

Introduction

Sepsis is one of the most critical and life-threatening syndromes worldwide. Sepsis occurs when a pathogen infects the body and an immunogenic dysregulated response occurs against the infection. Bacterial, fungal and viral infections are the primary etiological causes of sepsis (Grondman et al., 2020). More than 30 million people are affected annually by sepsis, with an incidence rate of 1% to 2% of all hospitalized patients globally (Huang et al., 2019). Unfortunately, sepsis represents a major cause of mortality in intensive care units (ICU) in hospitals, reaching 40% of the infected cases (Napolitano, 2018). However, early diagnosis and early initiation with the proper therapy can avoid the transition of sepsis to septic shock, multiorgan dysfunction syndrome (MODS) and death (Napolitano, 2018).

The Third International Consensus Definitions for Sepsis and Septic Shock (Sepsis-3) established the latest definition of sepsis in 2016. It states that sepsis is "a life-threatening organ dysfunction caused by a dysregulated host response to infection" (Singer et al., 2016). Thus, organ dysfunction is now strongly related to sepsis if it is not early diagnosed and correctly treated. Unfortunately, sepsis is not easily diagnosed since its apparent symptoms are considered familiar with various cardiovascular, respiratory, urinary tract and gastrointestinal diseases (Gauer, 2013). Patients come to emergency with symptoms like fever, chills, shortness of breath, high heart rate, confusion or disorientation and pain or discomfort (Centers for Disease Control and Prevention, 2021). Screening for sepsis and organ dysfunction is measured by Sequential (sepsis-related) Organ Failure Assessment (SOFA) score. The scoring system in the intensive care unit includes the patient's cardiovascular, respiratory, renal, hepatic and coagulation processes. The patient is addressed as a sepsis case with organ failure when SOFA ≥ 2 points increase from the baseline (Dolin et al., 2018). As SOFA score increases, the probability of mortality association increases (Singer et al., 2016). Besides, a septic shock is lately defined as a subset of sepsis with severe hypotension. It requires the use of vasopressors with fluid resuscitation to restore and keep the mean blood pressure above 65 mmHg and decrease a serum lactate level to be less than 2 mmol/L (Ljungström et al., 2019). There are also risk factors that increase the susceptibility to developing sepsis. Patients older than 65 years are 13 times more likely to develop sepsis and septic shocks, comorbidities, chronic illness, immunosuppression diseases or immunosuppressing medications, hospital-acquired infection after surgery and catheter insertion or other devices requiring an aseptic procedure (Gauer, 2013).

In Sweden, Ljungström et al. (2019) had conducted a population-based study on the annual incidences of sepsis based on sepsis-3 criteria in adult cases ≥18 years old. Retrospectively, it was found that the incidence of community-onset sepsis-3 838/100,000 (95% CI, 798–877). Moreover, prospectively, bacteremia was found in 13% of all admitted patients with an incidence of 203/100,000 per year (95%, CI 184–223) in Skaraborg hospital in the southwestern of Sweden. The results were considered from the highest reported values at the time of the study compared to other studies. Another study by Strandberg et al. (2020) studied the mortality rate of sepsis patients during the hospital stay in ICU. It was found that mortality was relatively high compared to non-sepsis ICU patients. The mortality for sepsis patients in 2016 was 30.5% at hospital discharge, 31.9% at 30 days, 38% at 90 days and in 2015, the mortality was 45.3% at 365 days. These results correspond to 15.6%, 17.1%, 20.7% and 26.7% for non-sepsis patients in 2016/2015. Therefore, early sepsis diagnosis is an urgent demand that can prevent the subsequent occurrence of serious complications and will help in declining morbidity and mortality rates in hospitals. In addition, the exorbitant expenses of additional treatment and prolonged hospital stay will be drastically decreased (Dolin et al., 2018).

Blood culture is still the gold standard of identification and diagnosis of sepsis. However, it is time-consuming and needs 2 to 3 days to decide whether the results are positive or negative. A positive result shows the growth of microorganisms after blood incubation in broth which confirms that the patient has bacteremia or fungemia in the bloodstream (Krans, 2018). Meanwhile, the negative result shows no microbial growth after incubation, which means that the patient is bacteremia-free and might have another kind of disease (Krans, 2018). Unfortunately, blood cultures can give false-positive results due to unintended contamination during sample extraction (Galleymore & Sahuquillo, 2019). Furthermore, in critically ill patients, early initiation with empirical antibiotics before blood culturing may show false-negative results due to the bacterial sensitivity to the antibiotic. In this scenario, the detection of true negative results will be quite challenging (Cheng, 2019). Also, there is a risk of developing antibiotic resistance due to the unnecessary administration of antibiotics for critically ill patients but with true negative results. Thus, blood culturing is not highly accurate or sensitive. Integration of early diagnostic technology is one of the key solutions to overcome all the disadvantages of blood culturing in septic and non-septic patients (De Waele & Dhaese, 2019). Technical-wise, one blood sample represents up to 20 mL of blood. Usually, blood is taken in two bottles with a total amount of 40 ml, one bottle is for aerobic bacteria and one is for anaerobic bacteria. The volume of blood withdrawal is a considerably large volume from a septic case suffering from loss of fluids and hypotension. This is another challenge in using blood culturing for sepsis patients (Pota et al., 2015).

Researchers at the University of Skövde in Sweden are currently trying to find a simple, early, rapid and advanced diagnostic method that can help clinicians in rapid and early diagnosis of a septic patient to save more lives. Scientists aim to achieve success in early diagnostics through biological biomarkers. Biomarkers are quantifiable characteristics that can be measured and analyzed scientifically in blood. Biomarkers act as an indicator of biological and pathological processes or pharmacological responses to medical treatment (Benz et al., 2016). Previously, a various number of biomarkers were studied for the diagnosis of sepsis like procalcitonin (PCT), neutrophil-lymphocyte count ratio (NLCR), C-reactive protein (CRP) and lactate. These biomarkers showed an improvement in the diagnosis of bacterial sepsis. However, they did not prove their complete accuracy and specificity in the early diagnosis of sepsis. For instance, CRP and PCT are now clinically used in routine diagnostic practice, but they have limited ability to differentiate in diagnosis between bacterial sepsis and other inflammatory diseases (Ljungström et al., 2017). In addition, the proinflammatory cytokine interleukin-6 (IL-6) is also measured in suspected sepsis cases. IL-6 shows elevation in serum level and can indicate the level of severity in septic shocks. Unfortunately, it was found that IL-6 is nonspecific to sepsis and it elevates with non-infectious diseases like trauma and stroke (Benz et al., 2016).

Recent studies have proposed circulating miRNAs as a suggested novel biomarker in sepsis identification which can build a new perspective in the diagnostic field (Benz et al., 2016). MicroRNAs (miRNAs) are non-coding RNA molecules with an approximate length of 18–24 nucleotides(Dave et al., 2019). They have an essential role in human gene expression control by acting as post-transcriptional gene regulators (Dave et al., 2019). The biogenesis of miRNAs starts with the transcription of miRNA genes inside the nucleus by RNA polymerase II to primary miRNA transcripts (pri-miRNA). The pri-miRNA is a double-stranded stem with a terminal loop structure and its length is between 500- 3000 nucleotides (Benz et al., 2016). The pri-miRNA is then cleaved by an enzyme called Drosha with its cofactor protein DGCR8 to precursor miRNA (pre-miRNA) of ~70 nucleotides in length (Correia et al., 2017). The pre-miRNA is then exported to the cytoplasm by exportin-5 protein. In the cytoplasm, the terminal loop structure of pre-miRNA is cleaved by Dicer enzyme resulting in a short double-stranded miRNA called miRNA duplex of around 17-24 nucleotides. Argonaute (AGO) proteins bind to the duplex, then one of the two miRNA strands degenerates, and the other strand becomes a mature miRNA forming RNA induced silencing

complex (RISC) (Benz et al., 2016). The inhibition and regulation of expression of a target gene occur by miRNA within the RISC, by base pairing to the 3′ untranslated regions (UTR) of the messenger RNA (mRNA) of the target gene (Jurcevic, 2015). The complementary binding of miRNA to the target mRNA can be partial or complete binding for gene expression regulation. Complete binding between miRNA and mRNA usually occurs in plants and results in degradation of the target mRNA. However, in mammals, partial binding between miRNA and mRNA generally occurs, and this results in inhibition of the translation process or its repression (Jurcevic, 2015) (Figure 1).

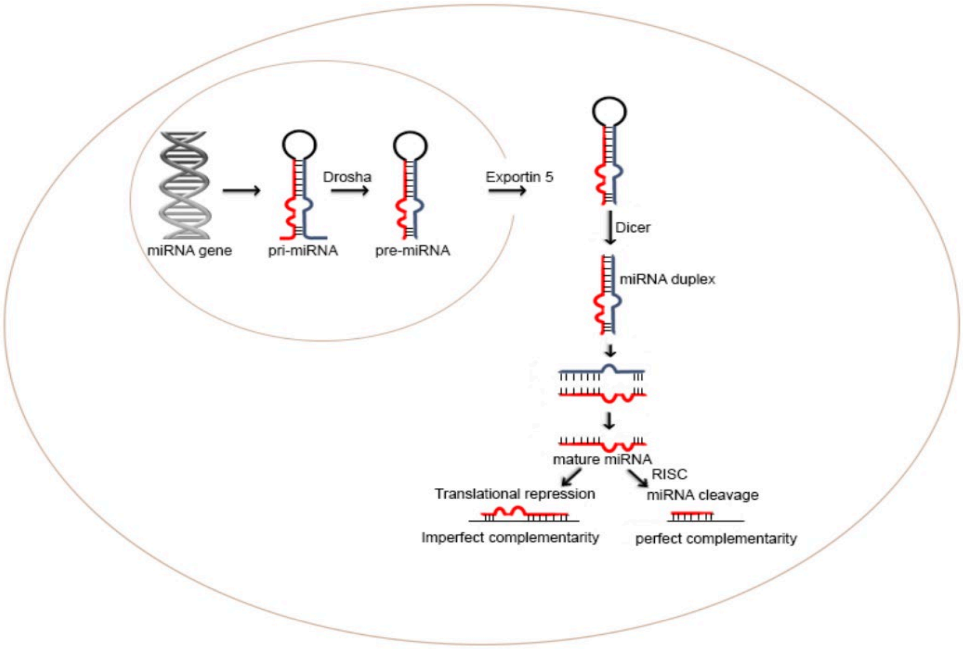

Figure 1. Copyrighted by Jurcevic, S. (2015). The Biogenesis of miRNA [Photograph]. From MicroRNA expression profiling in endometrial adenocarcinoma (Doctoral dissertation, Örebro university). https://www.diva-portal.org/smash/get/diva2:780763/INSIDE01.pdf. Used with permission.

Interestingly, miRNA genes probably present in only 1% of the human genome. However, they regulate up to 60% of all protein-coding genes (Benz et al., 2016). Therefore, the transcription of hundreds of mRNAs can be affected by one single miRNA (Benz et al., 2016). Previous studies had shown that miRNA expression varies and changes in numerous infections and diseases like cancer, liver, cardiovascular, autoimmune, and infectious diseases (Dave et al., 2019). These changes in expressions can act as an indicator for each specific disease. Moreover, miRNAs have another remarkable feature; they are stable in body tissues and extracellular biofluids such as urine, saliva, milk, blood plasma and serum (Correia et al., 2017). The high stability against degradation is due to their association with various RNA-binding proteins and binding to high-density lipoprotein or inclusion into microvesicles (Benz et al., 2016). Furthermore, the small non-complexed size of miRNA makes it less suspectable to modifications. (Benz et al., 2016). As a result, miRNAs are promising future biomarkers for diagnostics.

Studies have demonstrated miR-210 as a feasible biomarker for early or differential diagnosis of numerous disorders, particularly circulating miRNA-210(Ren et al., 2016). Usually, miRNA- 210 plays a vital role in mitochondrial metabolism, angiogenesis, cell proliferation, DNA repair and apoptosis (Bavelloni et al.,2017). However, Lin et al. (2019) found that miRNA-210 had shown upregulated expression levels in sepsis-induced acute kidney injury (AKI) in middle-aged and old patients. Moreover, miRNA-210 downregulation was an indicator for an increased survival rate of sepsis-induced AKI patients, which shows its beneficial effect in sepsis prognosis (Lin et al., 2019). Additionally, miRNA-210 has an oncogenic effect and it is significantly elevated in several

types of cancers as in breast, head and neck, lung and pancreatic cancer. Meanwhile, miRNA-210 overexpression in these diseases indicates a low prognosis (Bavelloni et al.,2017). Furthermore, studies had also shown the up-regulation of miR-210 induced by hypoxia in cancer cases and its potential to be used as a prognostic and diagnostic biomarker (Qin et al., 2014). However, in this study, synthetic miRNA-210 was used as a positive control but it might be included as a biomarker for early sepsis diagnosis in the future.

Generally, miRNAs would be a good candidate in the multi-marker panel in sepsis early detection and future point of care (POC) diagnostic tools (Dave et al., 2019). The idea of having a multi-marker panel is to have several biomarkers combined for one precise identification role, which will increase the accuracy in disease specification as in complex syndrome like sepsis (Casserly et al., 2011; Ljungström et al., 2017). However, the small size and similarity of sequencing between miRNAs require an array of advanced techniques for detection and quantification. Especially, miRNAs present in extremely low concentrations in the analyzing samples (Bartošík & Jiráková, 2018). Northern blot-based platforms, *in situ* hybridization, microarray, next-generation sequencing (NGS) and reverse transcription qPCR are methods that are most commonly used for miRNAs analysis (Dave et al., 2019).

Reverse transcription quantitative real-time PCR (RT-qPCR) is a popular and commonly used method for miRNA quantification and analysis. The reason for its spread is that it offers high sensitivity, high sample throughput, and has the ability to moderately multiplex targets (Correia et al., 2017). Furthermore, RT-qPCR is used to validate experimental results with high accuracy and precision. The concept is based on two fundamental steps. First is the reverse transcription step of the target miRNA to synthesis cDNA. Secondly, the quantitative amplification of the cDNA and detection are either by fluorescent dye-based or probe-based signals (Dave et al., 2019). There are many protocols and commercial kits that work with the same concept of RT-qPCR but with a different technique, e.g., TaqMan assays from Applied Biosystems/Fisher Thermo Scientific, miRCURY LNA qPCR from Exiqon and two-tailed RT-qPCR miRNA assays from TATAA Biocenter (Dave et al., 2019).

Samples with a limited target amount can be analyzed with two-tailed RT-qPCR (TATAA Biocenter) technology since it is considered a promising method of choice (Androvic et al., 2017). The two-tailed RT primers used in two-tailed RT-qPCR are different from regular primers. They have two hemiprobes complementary to different regions of the target miRNA (Androvic et al., 2017). Moreover, an oligonucleotide tether between the two hemiprobes is folded to form a hairpin-like structure to avoid unspecific reactions. This design increases the strength of binding between the two-tailed RT primer and the mature miRNA without binding to pri-miRNA or pre-miRNA and thus reinforces the reaction's sensitivity and specificity (Androvic et al., 2017).

Optimization of nucleic acid extraction significantly influences their detection and quantification afterward (Wright et al., 2020). In this project, total RNA extraction was done with two different methods manually and robotically via QIAcube (Qiagen) using the same kit miRNeasy® Serum/Plasma Advanced kit protocol (2020). The reason for using the same kit is to assess which method can yield better quality and quantity of miRNAs as biomarkers for sepsis diagnosis. The extraction method with higher evaluation can be used as a benchmark in clinical laboratory diagnostic work. QIAcube (Qiagen) is an automated machine used in laboratories to purify proteins and nucleic acids with high technology. It can work with different preinstalled kits that are cheaper than kits based on magnetic bead technology (McGraw et al., 2014). A study by McGraw et al. (2014) with QIAcube had shown that QIAcube could purify up to 12 proteins or nucleic acids seamlessly without massive manual interference. Working with automation can save time, effort, decrease the sample volume required for diagnosis and reduce the risk of cross-contamination (Tan & Yiap, 2009). This study aims to assess the quality of miRNAs by the two

extraction methods and optimize the two-tailed RT-qPCR technology to detect and quantify miRNAs as biomarkers in a multi-marker panel for the early diagnosis of sepsis.

Aim

This study is one of many ongoing projects in the research field of sepsis led by Dr. Anna-Karin Pernestig, a Senior Lecturer in Biosciences at the University of Skövde in Sweden. The study is underlying an important project which is "Future diagnostics of sepsis". The main question here is how to achieve an early and accurate diagnosis of sepsis in the future ?, particularly "Are miRNAs possible candidate as biomarkers in a multi-marker panel for early sepsis diagnosis ?". Pernestig and her team aim to work on proteins, microRNA and the vital parameters as biomarkers to identify patients with bacterial sepsis earlier than expected nowadays. Thus, increase the chance of patient survival and decrease comorbidities. The aim includes creating a multi-marker panel with data mining for designing an early, rapid and user-friendly diagnostic kit for bacterial sepsis. Previous projects at the University of Skövde had worked and developed prospective multi-marker panels based on miRNAs and routine clinical biomarkers. This study worked with the same aim but with different objectives. The first objective is to extract miRNAs from self-assessed healthy donors' human plasma, manually and robotically by QIAcube (Qiagen), using miRNeasy® Serum/Plasma Advanced Kit (Qiagen). Secondly, to compare the quality of the extracted miRNAs by measuring the concentration and purity of the RNA eluates extracted by both methods. The reason for comparing the quality is to check which method can be considered as a benchmark for the best practice laboratory work in miRNAs extraction in the future. Lastly, to optimize the two-tailed RT-qPCR technology for precise miRNAs detection and quantification after the extraction process. The two-tailed RT-qPCR technology is newly used on endogenous miRNAs in human plasma at the University of Skövde and needs to be optimized before using it in the validation of the multi-marker panel.

Thus, the "Future diagnostics of sepsis" project can help clinicians to make the correct decision in patients' treatment. Either by prescribing the right antibiotic at the right time if the patient has bacterial sepsis or avoiding overuse of an unwanted antibiotic if the patient has another disease excluding sepsis.

Materials and methods

Study outline

This project was working on total RNA extraction and miRNAs detection. The study was performed on the naturally existing miRNA in self-assessed healthy adult donors' plasma and spiked-in healthy donors' plasma with synthetic miRNA-210 (integrated DNA Technologies). The number of samples for the whole thesis work was 48 samples (Appendix A1). The workflow is explained in (Figure 2).

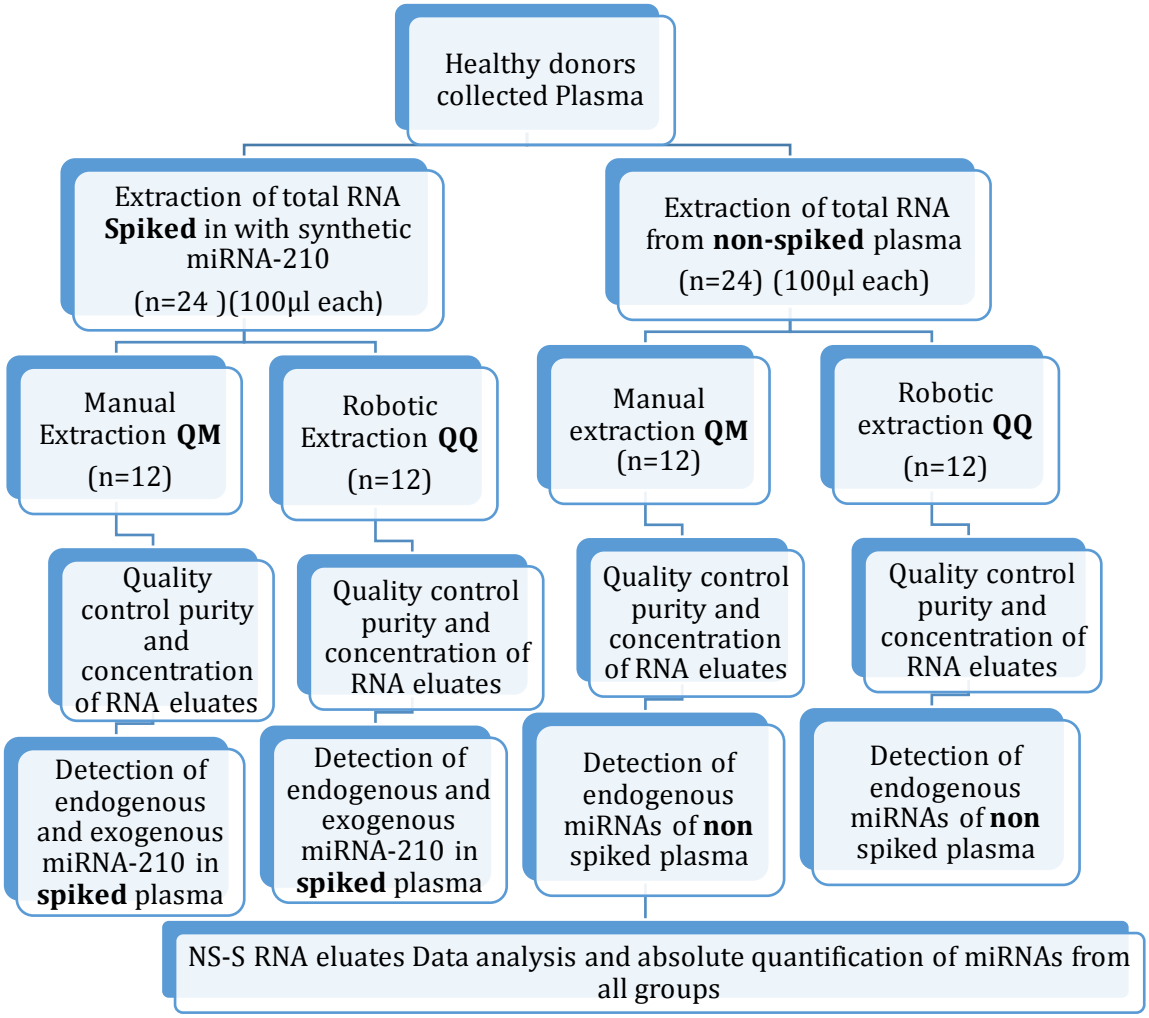

Figure 2. The workflow of the whole thesis project. QM stands- Qiagen Manual extraction (n=24), QQ-Qiagen QIAcube extraction (n=24). The number of samples was twelve in each of the four groups (n=12). The miRNeasy® Serum/Plasma Advanced kit protocol from Qiagen was used for both extraction methods. Each RNA eluate extracted sample was around 18 µl. Non-spiked and spiked RNA eluates were referred to as NS-S RNA eluates. Detection of miRNAs was done by two-tailed RT-qPCR technology. Data analysis and absolute quantification of all the detected miRNAs were performed.

Plasma collection

In 2014, blood was collected from healthy self-assessed adult donors. The blood collection was done in Unilabs, Skaraborg Hospital, Skövde in Sweden. The whole venous blood was collected in 6 ml K2EDTA Vacuette Tubes (Greiner Bio-One). Blood samples were centrifuged at 2000 x g at room temperature for ten minutes. After plasma separation from the other blood components, Plasma was transferred and divided into other tubes. Each tube has 300 µl of plasma. Plasma tubes were stored at the University of Skövde in a -80 °C freezer.

Extraction and purification of miRNAs

Manual total RNA extraction, including miRNA

Four 300 µl plasma coded tubes were placed in 37°C water bath VWB 2 (VWR) for 5 minutes for thawing. The tubes were then divided into twelve plasma samples, 100 µl each in a 2 ml microcentrifuge tube (Eppendorf). Extraction was performed following miRNeasy®

Serum/Plasma Advanced kit Protocol 2020: Purification of Total RNA, including miRNA, from Serum and Plasma (Qiagen) with minor changes. This protocol assumes the starting plasma amount is 200 µl. However, this project worked on 100 µl of plasma for each sample. Thus, 30 µl of RPL buffer and 10 µl of RPP buffer were used instead of 60 µl RPL and 20 µl RPP respectively. Spike in with synthetic miRNA-210 (Integrated DNA Technologies) diluted in RNase free water (Qiagen) 1:10 folds were done to get 10^5, 10^6, 10^7 copies of miRNA-210 (integrated DNA Technologies). The spike-in step had been done before the extraction process was completed. Subsequently, four plasma samples were spiked in with each copy number (manually and robotically) (Appendix A2). For the spike-in step, 2 µl of plasma were removed and replaced with 2 µl of synthetic miRNA-210 with different copies (Appendix A2). The extraction of non-spiked plasma was carried out following the same steps of spiked-in plasma samples, excluding the spike in step number "four" in the protocol. NS-S RNA eluates from the manual extraction process were around 18 µl.

Robotic total RNA extraction including miRNA

The robotic extraction via QIAcube (Qiagen) was performed to spiked and non-spiked plasma (Appendix A1). Similarly, as the manual extraction, the automated extraction was done from healthy donor's plasma samples stored in a -80 °C freezer with code numbers. Before the extraction process, tubes were placed in 37 °C water bath VWB 2 (VWR) for 5 minutes for thawing. Each tube sample was around 200-300 µl. Therefore, each tube was divided into plasma samples of 100 µl each in a 2 ml microcentrifuge tube (Eppendorf). The miRNeasy® Serum/Plasma Advanced kit Protocol 2020: Purification of Total RNA, including miRNA, From Serum and Plasma (Qiagen), was used in this process for both spiked and non-spiked plasma samples. Manual steps were followed from step number one to six in the protocol. Spike-in was done in step number "four" in the protocol with different copies of synthetic miRNA-210 before extraction is completed (Appendix A2). As in the manual extraction process, for spiking the plasma samples, 2 µl of plasma were removed and replaced with 2 µl of synthetic miRNA-210 with different copies. For non-spiked plasma samples, step number "four" in the protocol was skipped. QIAcube (Qiagen) performed the rest of the protocol steps from step number seven to 13, and NS-S RNA eluates of 18 µl were produced by QIAcube (Qiagen) machine.

Quality Control

Purity and concentration

After the extraction process, all NS-S RNA eluates were measured for their quality and quantity (Figure 2). The purity of total RNA extracted was measured by a Ds-11 spectrophotometer (Denovix), which has the same function as Nanodrop. The concentration of small RNA was measured by Qubit 3.0 Fluorometer (ThermoFisher Scientific) using Qubit® microRNA Assay kit.

Hands-on time and turn-around time

The two methods used for RNA extraction were assessed for the estimated times needed for the operation. First, hands-on time was measured when manual and handwork were performed, excluding fixed times in the protocol like incubation and centrifugation time. Besides, the turn-around time was also measured, which is the time needed to accomplish the whole extraction steps. These times were calculated using a digital watch during the extraction of six non-spiked plasma samples from each method manually and robotically.

Detection and quantification of miRNA by two-tailed RT-qPCR

This method was used as a downstream application to the extracted miRNAs. The best way to optimize the qPCR assay is to run a serial dilution to the template, which is synthetic miRNA-210 (Integrated DNA Technologies). A linear standard curve was obtained from the results (Bio-Rad laboratories, 2006).

Standard curve determination

A serial dilution in nuclease-free water was done 1:10 folds from the original template of 2 µl synthetic miRNA-210 (10^{14}) copies (Integrated DNA Technologies). The diluted miRNA-210 copies were eight samples from 10^{12} to 10^{5} copies of miRNA-210 (integrated DNA Technologies). Serial dilutions were used to obtain a standard curve. Absolute quantification of NS-S RNA eluates was performed on the most optimized standard curve.

Reverse transcription (RT) of miRNAs and cDNA synthesis

For each standard curve, the eight diluted miRNA-210 templates 10^{12},10^{11},10^{10},10^{9},10^{8},10^{7},10^{6} and 10^{5} copies were converted to cDNA templates by reverse transcription using GrandScript cDNA FreePrime kit (TATAA Biocenter). The preparation and the technique were followed as the protocol with some modification in the volumes of the master mix (Table 1). Besides, four randomized no reverse transcriptase (NRT) samples were prepared as negative controls. Samples were then run in the PCR machine (PTC-200) for 45 min at 42 °C, followed by 5 min at 85 °C and cooled at 4 °C as per GrandScript cDNA FreePrime kit, the protocol for two-tailed priming (TATAA Biocenter). Each reaction for the standard curves was around 20 µl. Therefore, the volumes in (Table 1) were doubled for standard curve cDNA synthesis. After the RT reaction completion, Samples were then stored at -20 °C for qPCR afterward. The process was done and repeated for standard curve generation and for NS-S RNA eluates used in qPCR. However, the RT reactions of NS-S RNA eluates were 10 µl each reaction, not 20 µl as in standard curves RT reactions.

Table 1. GrandScript cDNA FreePrime protocol to convert miRNA to cDNA in 10 µl reaction.

Component	Volume (µl)
GrandScript FreePrime Reaction Mix (5x)	**2**
GPS enhancer (10x)	**1**
GrandScript RT Enzyme*	**0.5**
Two-tailed primer (0.2 µM) **	**2.5**
RNA plasma template	**4**
Nuclease free water	**-**
Total	**10**

*NRT controls were prepared by adding 0.5 µl of nuclease-free water to the master mix instead of 0.5 µl GrandScript RT Enzyme.

**Two-tailed primer (10 µM) was diluted with nuclease-free water prepared by the Millipore Direct- Q3 Water Purification System (ThermoFisher) to get the final concentration (0.2 µM)

Quantitative PCR (qPCR)

The resulted cDNA templates from the RT reactions of standard curve preparation and the NS-S RNA eluates were quantified by following the two-tailed RT-qPCR protocol using TATAA SYBR® GrandMaster® Mix (TATAA Biocenter)(Table 2). A reference dye ROX (Biotium) was additionally added to normalize the variation between the wells of qPCR reactions (Biotium,2021). Negative controls were no template control (NTC) and NRT. NTC was prepared by adding 2 µl nuclease-free water to the master mix instead of 2 µl of cDNA template. Besides, all the samples in the plate were placed with technical replicas as triplets. Before running the qPCR reactions, the 96 wells plates (Applied Biosystems) were vortexed by Analog vortex mixer (VWR) and centrifuged in a plate centrifuge (Nippon Genetics Europe) for 10 seconds. Plates were then in the qPCR machine 7300 Real-Time PCR system (Applied Biosystems). The cycle protocol was set to 95°C for 30 sec. in stage 1. In stage two, 40 cycles were running at 95°C for 5 sec., 60°C for 15 sec. and 72°C for 30 sec. The cycle run was as per the protocol but with some modifications.

Table 2. Master mix components of qPCR by SYBR grandmaster mix kit from TATAA Biocenter.

Component	Volume (µl)
TATAA SYBR® GrandMaster® Mix (2x)	5
Forward primer (10 µM)	0.2
Reverse primer (10 µM)	0.2
cDNA	2
Nuclease Free Water	2.4
ROX dye (25 nM)	0.2
Total	10

After the qPCR was over, data was collected, the standard curve was optimized and absolute quantification was done. Moreover, melting curves of qPCR amplification were analyzed, and all the tested samples were analyzed with statistical analysis.

Absolute quantification of miRNAs

Absolute quantification was done by relating the mean Cq of the technical triplicates from the tested samples to the standard curve obtained by two-tailed RT-qPCR (TATAA Biocenter). Absolute quantification was done to determine the unknown concentrations of the miRNAs. Once miRNAs quantification was successful from spiked RNA eluates, quantification was done on naturally existing miRNAs in human plasma, i.e., from plasma that had not been spiked with any synthetic miRNA. The process was carried out on a few selected NS-S RNA eluates extracted manually and robotically by QIAcube (Qiagen). The NS RNA eluates used in absolute quantification were named NS_3, NS_4, NS_5, NS_6, NS_7, NS_8, NS_9, NS_{10}, NS_{11}.

Data analysis

To analyze the results, statistical analysis was conducted. Analysis was performed using RStudio software Version 1.4.1103 (RStudio, PBC). First, before comparing the data, Shapiro-Wilk normality test was done to check if the data is normally distributed or not. The significance level was set to ($p = 0.05$). Next, the Mann Whitney U test was used to compare robotic and manual extraction concentration and purification as the data were not normally distributed. Finally, the standard curve determination and absolute quantification were done with Microsoft Excel Version 16.47 (Microsoft).

Results

Quality control of RNA eluates results

Once the extraction of NS-S RNA eluates was done manually and via QIAcube, the concentration of the small RNA and the purity of the total RNA extracted were measured (Table 3).

Table 3. Mean values with the standard deviation of small RNA concentrations and total RNA purity absorbance ratio 260/280 for spiked and non-spiked RNA eluates manually and robotically.

Sample group	Small RNA concentration Mean (ng/µl) ±SD	Total RNA $A_{260/280}$ ratio Mean ±SD
Spiked QM (n=12)	0.47±0.65*	2.20± 1.56
Spiked QQ (n=12)	1.22± 1.01	1.24± 0.35
Non-spiked QM (n=12)	0.10±0.19*	1.43± 0.33
Non-spiked QQ (n=12)	0.48±0.49*	0.76± 0.15

*The undetected values by Qubit 3.0 (ThermoFisher) were replaced with a constant = limit of quantification of Qubit (0.05 ng/ µl)/√2. Detailed results are in Appendix B.

Quality control of the extraction methods and concentration of small RNA results

Concentration outlier detection

Qubit 3.0 Fluorometer (ThermoFisher Scientific) was unable to read the concentration in 79.2% (19/24) of NS-S RNA eluates that were manually extracted using miRNeasy® Serum/Plasma Advanced kit (Appendix C1). Meanwhile, Qubit 3.0 Fluorometer (ThermoFisher Scientific) was unable to read the concentration of small RNA in 16.7% only (4/24) NS-S RNA eluates extracted via QIAcube using the same kit (Appendix C1). Although there is a massive difference between the number of eluates measured, boxplots were plotted to see the outliers in the concentration reading data. The undetected values were placed with a constant that was calculated by the limit of quantification of Qubit microRNA Assay (0.05 ng/µl)/√2 =0.04 (data not shown).Therefore, each of the 19 undetected NS-S RNA eluates in QM data were replaced with a constant = 0.04 and the four undetected NS-S RNA eluates from the QQ data were also replaced with 0.04 constant. Thus, all the 48 samples extracted manually and robotically were used in the analysis and outliers were detected (Figure 3).

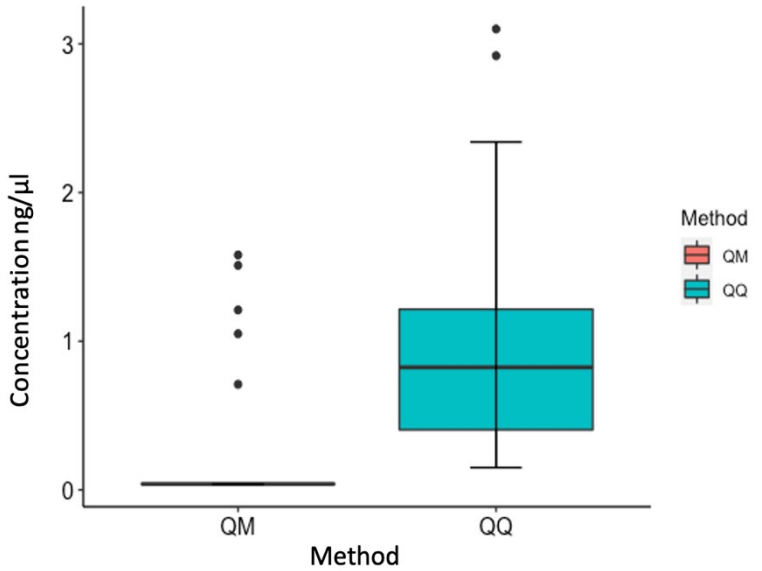

Figure 3. Box plots of small RNA concentrations extracted manually and by QIAcube. Each box present interquartile range (IQR). The horizontal black line in each box presents the median of the data. The vertical line presents a 95% confidence interval (CI). A) QQ shows two points are more than 1.5 IQR away from the

box edge and presented as outliers. QM presents all the NS-S RNA eluates extracted manually (n=24) and QQ presents NS-S RNA eluates extracted by QIAcube (n=24).

Normality test and significance test of concentration measurements

The Shapiro-Wilk normality test was carried out to check if the QM and QQ data were normally distributed. The significance level was set to (p=0.05). The small RNA concentration extracted by QM p-value <0.001 and the small RNA concentration extracted by QQ also p-value <0.001. Therefore, the Mann Whitney U test (Wilcoxon rank-sum test), a non-parametric test that was used since the data was not normally distributed. The test was used to see if there was a significant difference in small RNA concentration between the QM and QQ extractions. The significance level was set to (p=0.05). The resulted p-value <0.0001, which is less than the significance level (Figure 4). The results emphasized a clear significant difference between the concentration of the small RNA extracted by QM and the concentration of small RNA extracted by QQ (Figure 4).

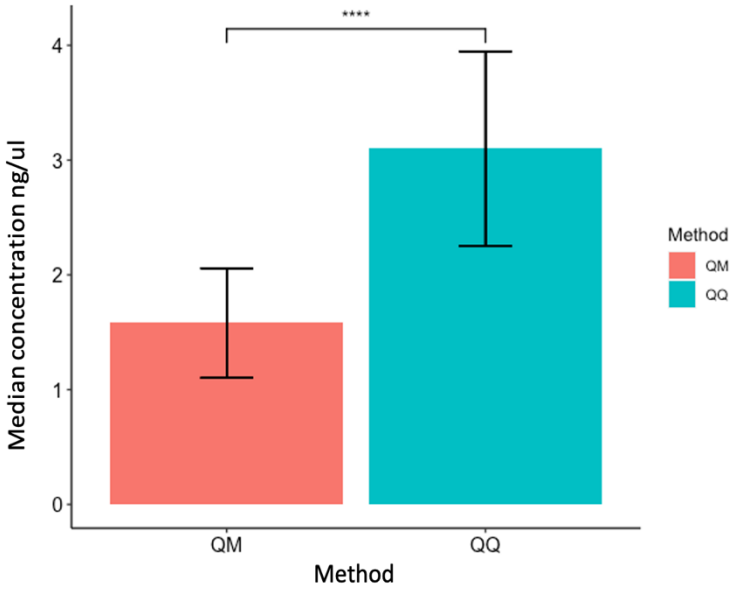

Figure 4. A Bar chart shows the medians of small RNA concentration extracted by two methods QQ and QM. Error bars indicate a 95% confidence interval (CI). Statistical significance was obtained by the Mann Whitney U test (W = 489, p-value <0.0001). The number of samples in the QM group (n=24) and the number of samples in the QQ group (n= 24).

Quality control of the extraction methods and purity of total RNA results

The absorbance ratio 260/280 was used to compare the purity of NS-S RNA eluates from QIAcube and manual extractions. The analysis was done in a similar way to the concentration of small RNA analysis.

Purity outlier detection

All the eluates extracted manually and by QIAcube gave results (Appendix C2). However, the boxplot showed few points as outliers (Figure 5A). One point which was hugely deviated in the QM purity result was removed (Figure 5B).

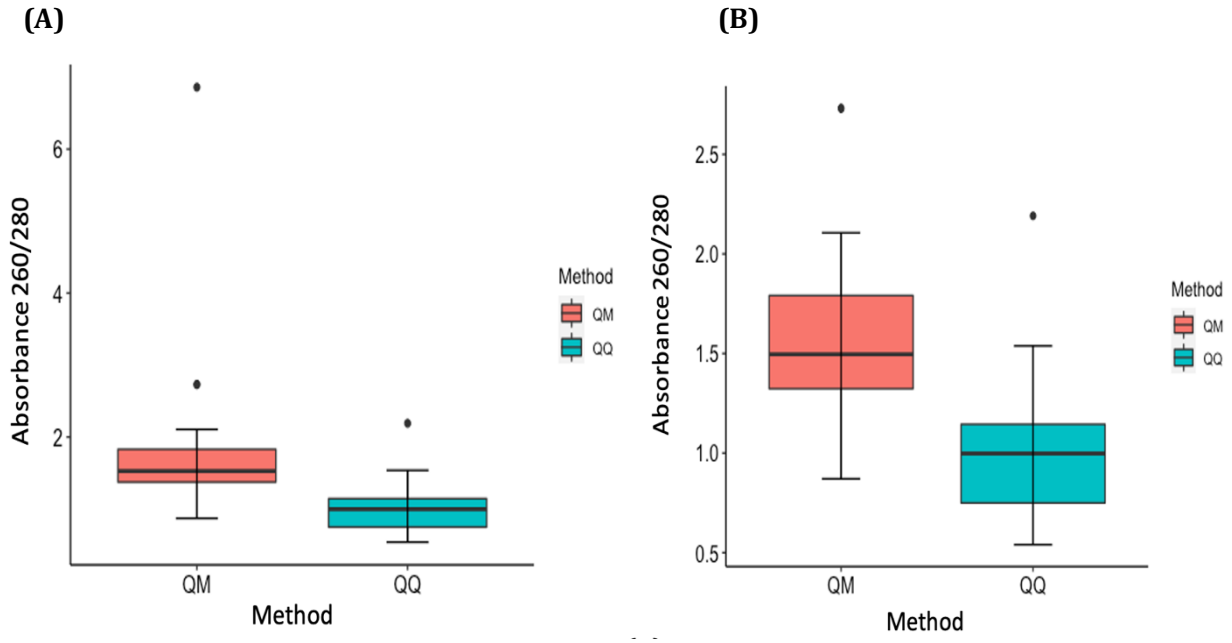

Figure 5. Boxplots of total RNA purity absorbance ratio 260/280 from NS-S RNA eluates extracted by QM and QQ. Each box present interquartile range (IQR). The horizontal black line in each box presents the median of the data. The vertical line presents a 95% confidence interval (CI). A) QM shows two points are more than 1.5 IQR away from the box edge and presented as outliers. QQ showed one point above the confidence interval error bars and presented as an outlier. B) box plots after removing one outlier point above 6 in QM values (n=23). QQ outlier was not removed (n=24).

Normality test and significance test of purity measurements

The data analysis was carried out on total RNA purity absorbance ratio of 260/280. One outlier was removed from QM values, and the Shapiro-Wilk test was done to check the normality of the data. The significance level was (p=0.05). The total RNA purity extracted by QM p-value = 0.05 and the total RNA purity extracted by QQ p-value = 0.005. Thus, the Mann-Whitney U test was selected to compare the two variables, QM and QQ, to test if there was a significant difference between total RNA purity resulted from both methods. The significance level was p= 0.05. The test resulted in (P<0.0001) (Figure 6). Therefore, the p-value was less than the significance level, which means that there was a significant difference between the total RNA purity extracted by QM and QQ (Figure 6).

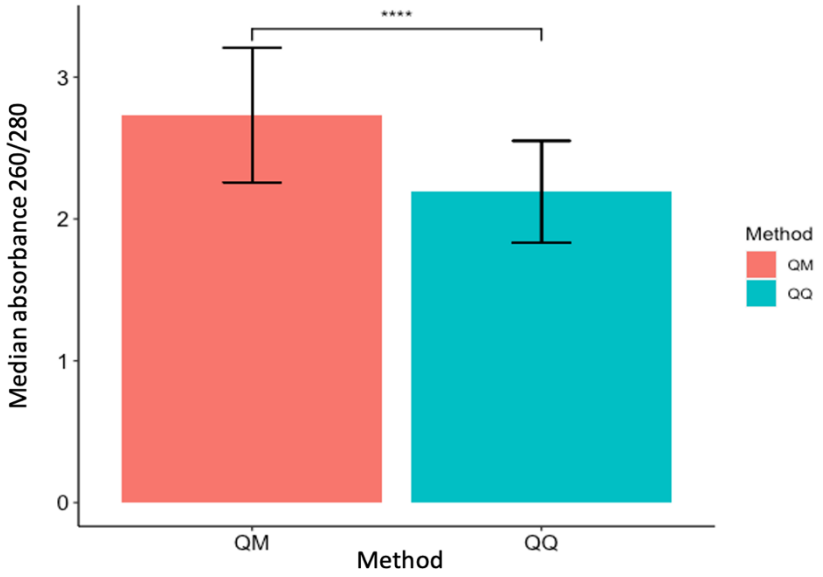

Figure 6. A Bar chart shows the medians of absorbance ratio 260/280 of NS-S RNA eluates extracted by two methods QQ and QM. Error bars indicate a 95% confidence interval (CI). Statistical significance was obtained by the Mann Whitney U test (W= 68, p-value <0.0001). The number of samples in the QM group (n=23) and the number of samples in the QQ group (n= 24).

Hands-on time and turn-around time for manual and robotic RNA extractions

The RNA extraction time by the two methods was measured for six non-spiked plasma samples (Table 4). The average time needed for one sample extraction was also calculated by dividing the time consumed from these six non-spiked samples from each method by 6 to know the average times needed if only one sample was being extracted (Table 4).

Table 4. Hands-on time and turn-around time required for RNA extraction process manually and by QIAcube.

Method	Hands-on time (minutes) (n=6)	Hands-on time (minutes) (n=1)	Turn-around time (minutes) (n=6)	Turn-around time (minutes) (n=1)
Manually	47	8	90	15
QIAcube	21	3.5	44	18.5*

*QIAcube works with a minimum of two samples per run and the minimum approximate time is around 15 minutes.

Detection and quantification of miRNAs by two-tailed RT-qPCR Results

The best standard curve was selected and used to quantify detected miRNAs in NS-S RNA eluates by absolute quantification.

Standard curve optimization

A standard curve was constructed by plotting the average Cq values of triplets for different copy numbers of miRNA-210 amplifications on the y-axis against the logarithmic concentration of the miRNA-210 with different copy numbers on the x-axis (Figure 7). In addition, the recommended parameters for an optimized qPCR were evaluated (Table 5).

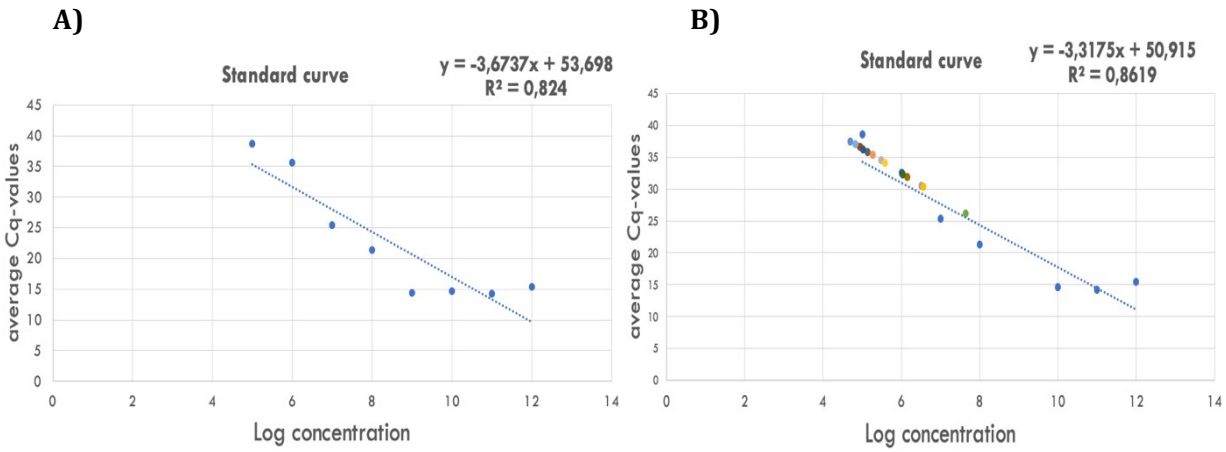

Figure 7. Standard curves determined from synthetic miRNA-210 (Integrated DNA Technologies) serial dilution showing results and linearity before and after omitting outliers. (A) a standard curve before the removal of 10^6 and 10^9 copies of miRNA-210 with slope m= -3.67 and R^2= 0.824. (B) standard curve after removing 10^6 and 10^9 copies of miRNA-210 shows the better slope and linearity m= -3.3175 and R^2= 0.861 with absolute quantification for the unknown concentration of selected NS-S RNA eluates.

Table 5. Shows the standard curve data before and after removing 10^6 and 10^9 copies of miRNA-210.

Criteria	Standard curve before outlier removal	Standard curve after outlier removal
Coefficient of determination (R^2)	0,824	0,860
Slope (m)	-3,6737	-3,3175
Amplification efficiency (E)*	1,87	2,00
Efficiency percentage (%)	87 %	100%

* The amplification efficiency was calculated based on the following equation $= 10^{-1/m}$ and converted to a percentage with $E\% = (E - 1) \times 100$.

Absolute quantification of miRNAs in NS-S RNA elutes

Absolute quantification was done using the optimized standard curve to determine the copy concentration of all miRNAs in spiked RNA eluates and the copy concentration of endogenous miRNAs in NS RNA eluates (Figure 7B). All the tested NS-S RNA eluates were quantified by two-tailed RT-qPCR (TATAA Biocenter) in triplets as technical replicas. Quantification was on samples extracted with QM and with QQ (Figure 8). Generally, six spiked RNA eluates were quantified (n=6). Three with different copy numbers of synthetic miRNA-210 (Integrated DNA Technologies) extracted manually QM (n=3) and another three eluates with different copy numbers extracted by QIAcube QQ (n=3) (Table 6A). Spiked RNA eluates were used as a positive control to ensure the success of the quantitative amplification by two-tailed RT-qPCR (TATAA Biocenter). In total, 18 NS RNA eluates were evaluated where results from QM (n=9) and QQ (n=9) extractions were compared. The NS RNA eluates were selected randomly from QM and QQ extraction methods (Figure 8B). Unfortunately, three out of nine samples (NS_4, NS_5 and NS_{11}) in both QQ and QM did not show amplification of miRNAs by qPCR or showed high variance between the replicas (data not shown). Thus, these three samples were excluded from applying absolute quantification. However, the rest of the six samples showed mixed results from QM (n=6) and QQ (n=6). Thus, the total number of NS RNA eluates included in the analysis (n=12) (Table 6B).

Table 6. Absolute quantification of miRNAs showing log concentration values. (A)Absolute quantification of synthetic miRNA-210 and endogenous miRNAs in spiked RNA eluates. (B) Absolute quantification of endogenous miRNAs in NS RNA eluates.

(A)

Spiked RNA eluates miRNA-210 with endo. miRNA	Mean Cq ± SD	Log Concentration
QM (10^5)	36.44±1.01	$10^{4.98}$
QQ (10^5)	30.52±0.35	$10^{6.52}$
QM (10^6)	30.36±0.49	$10^{6.56}$
QQ (10^6)	32.53±0.44	10^{6}
QM (10^7)	26.22±0.48	$10^{7.64}$
QQ (10^7)	32.44±1.49	$10^{6.02}$

(B)

NS RNA eluates with endogenous miRNA	Mean Cq ± SD	Log Concentration
NS 3 QM	NA*	NA
NS 3 QQ	36.60± 1.43	$10^{4.94}$
NS 6 QM	35.86±0.96	$10^{5.13}$
NS 6 QQ	31.91±0.09	$10^{6.16}$
NS 7 QM	36.27± 0.72	$10^{5.02}$
NS 7 QQ	32.35±0.32	$10^{6.04}$
NS 8 QM	37.53± 0.34	$10^{4.70}$
NS 8 QQ	35.35±1.03	$10^{5.26}$
NS 9 QM	36.14±0.68	$10^{5.48}$
NS 9 QQ	34.15±1.23	$10^{5.56}$
NS 10 QM	NA	NA
NS 10 QQ	37.06± 0.462	$10^{4.82}$

* NA data is not available since Cq values are undetermined by two-tailed RT-qPCR.

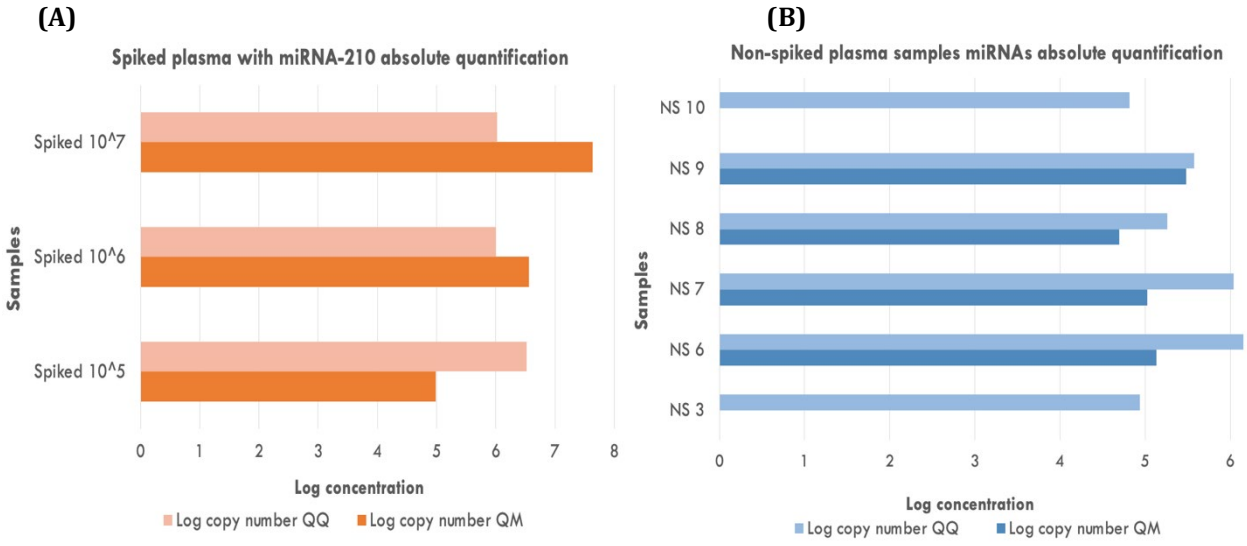

Figure 8. A graphical presentation of absolute quantification results of manually and QIAcube extracted miRNAs. (A) absolute quantification of miRNAs in spiked-in RNA eluates with miRNA-210 (n=6). (B) absolute quantification of miRNAs in non-spiked RNA eluates (n=12).

Melting curve analysis

All the tested NS-S RNA eluates, robotically and manually extracted, showed one peak during the two-tailed RT-qPCR (TATAA Biocenter) amplification process (Appendix D). In addition, the negative controls NTC and NRT in most plates did not show any amplification (Appendix D). Thus, this emphasizes that the tested samples were uncontaminated with secondary products, and the validity of the two-tailed RT-qPCR results was ensured.

Discussion

One of the operational considerations that have an impact on the quality of miRNA results is the choice of the miRNA extraction kit. The selection of the kit should be based on how easy to use the protocol and the time frame needed to accomplish the protocol with the thoughtfulness of different individual technical skills. These criteria are essential, especially when working with experimental procedures with high throughput (Wright et al., 2020). Wright et al. (2020) compared in a study between six different kits for miRNAs extraction. Across the operational factors, miRNeasy® Serum/Plasma Advanced Kit (Qiagen) was one of the top three kits. The kit is an alternative to the miRNeasy Serum/Plasma Kit with the same yield of RNA and better quality (Qiagen, n.d.). This is because miRNeasy Serum/Plasma Advanced Kit is a phenol-free protocol that uses MinElute spin column technology without a vacuum pump. Phenol residues usually affect the purity of extracted RNA. Contamination with phenol can lead to under or overestimation of the actual concentration in extracted samples and thus impact negatively on RT-qPCR results (Unger et al., 2019). Nordén (2020) had assessed different miRNA isolation kits and found that the concentration of miRNAs with miRNeasy Serum/Plasma Advanced Kit was slightly higher than miRNeasy Serum/Plasma Kit, which resulted in better amplification results in qPCR.

Since miRNAs are remarkably stable and exist in extracellular biofluids as discussed before, this project worked on miRNAs in plasma cells, not in blood serum. A study by Dufourd et al. (2019) showed that the extraction of miRNA from blood plasma in rats and humans was more detectable than extraction from blood serum. Furthermore, miRNA concentrations of serum are agitated more than plasma by red blood cell hemolysis and coagulation factors which may release unspecific miRNAs to the serum resulting in inaccurate detection (Eslamizadeh & Akbari, 2020). The volume of plasma used in this study was 100 µl not 200 µl as recommended by miRNeasy Serum/Plasma Advanced Kit (Qiagen) for two reasons. First, the plasma used in this study was for clinical diagnostic purposes. Therefore, tested samples should be in minimal reliable volumes (Wright et al.,2020). Secondly, A study by Öberg (2020) showed that 100 µl of plasma is efficient as a starting volume for miRNAs extraction and applicable for downstream application for miRNAs detection by RT-qPCR. Thus, the use of 100 µl of plasma in this study was fruitful and showed valuable results that will be discussed later below.

Spiked-in RNA eluates with synthetic miRNA-210 (integrated DNA Technologies) were used as a positive control for non-spiked RNA eluates extraction and detection during the thesis project. Spike-in means adding a known amount of exogenous synthetic miRNAs into samples that contain variable amounts of miRNAs that the study aims to detect and analyze (Moldovan et al., 2014). The idea of spike-ins is to measure miRNA efficiency of isolation and to control sample to sample variations. Thus, it is a perfect approach for microRNAs normalization (Scheffer et al., 2014). Furthermore, It is crucial to monitor technical factors that influence the final miRNA levels, and that can be done by using spike-ins (Brunet-Vega et al., 2015). In this study, the success of miRNA extraction and quantification from spiked RNA eluates gave an impulse to continue with miRNAs isolation and quantification from the NS RNA eluates.

Quality control of the extraction methods and concentration of small RNA analysis

One of the biggest challenges during small RNA concentration measurement was the undetectability of 11/12 samples of NS QM eluate, 4/12 of NS QQ eluates and 8/12 of spiked QM eluates (Appendix B). The undetected values were replaced with a constant for statistical analysis (Table 3). Generally, spiked RNA eluates were more detectable than NS RNA eluates (Appendix B). It was also noticed that most of the undetected measures were QM extracted and few were QQ extracted (Appendix C). Standard deviation was measured to see how the data was scattered from the mean (Table 3). A low standard deviation close to zero means that the data was close to the mean and more accurate, while a high SD indicates low precision of the data (Lee et al., 2015; National Institutes of Health, n.d.). On the other hand, Qubit 3.0 Fluorometer (ThermoFisher Scientific) can accurately read initial sample concentration from 0.05 ng/µl to 100 ng/µl (Qubit® microRNA Assay Kits, 2015). The undetectable concentrations were showing a message on the screen of Qubit 3.0 "sample is too low", which means that the concentration of small RNA in the eluates is less than 0.05 ng/µl but does not mean that small RNA including miRNAs do not exist. Thus, these eluates were used in the downstream application by two-tailed RT-qPCR (TATAA Biocenter) and miRNAs were successfully detected and quantified.

Missing data is always an obstacle in scientific research and a barrier to performing different tests for significance between different variables. To solve this, every undetectable value below the Limit of Quantification (LOQ) of Qubit 3.0 Fluorometer (ThermoFisher Scientific) was replaced by a constant by using $LOQ/\sqrt{2}$ equation (Succop et al., 2004). Limit of Quantification is the lowest concentration of an analyte that can be detected accurately and precisely (Armbruster & Pry,2008). The limit of quantification can be equivalent to the limit of detection (Armbruster & Pry,2008). The detection limit of Qubit (ThermoFisher Scientific) is 0.05 ng/µl (Garcia-Elias et al., 2017). Therefore, after using the equation, a constant of 0.04 was placed instead of the missing data to be used in data analysis for outliers' detection. Besides, the constant was used to test the significant difference in the concentration of small RNA in NS-S RNA eluates extracted by QM and QQ.

Outlier and significance test analysis of concentration results

There were five outliers in the QM data out of 24 samples used in the analysis (Figure 3). However, these outliers were the true values measured and read by Qubit 3.0 (ThermoFisher Scientific). Therefore, these outliers were kept, although there was a huge bias towards zero due to the existence of the constant (0.04) of the undetected values. Meanwhile, QQ data showed two outliers that had values greater than 2.5 ng/µl (Figure 3). The two values were also kept, and none of them was removed. Lindeberg (2020) worked on the detection and quantification of miRNAs in non-spiked plasma extraction manually and by QIAcube (Qiagen). The study had 2/10 of QQ values above 2.5 ng/µl during the concentration analysis by Qubit 4.0 (ThermoFisher Scientific), which justifies the reliability of two outlier values of the QQ in this study. In addition, the concentration of the QQ outliers might be a bit higher than other values, as these two samples were spiked-in with synthetic miRNA-210(Integrated DNA Technologies). Therefore, all the 48 NS-S RNA eluates were tested if there were any significant differences between the two methods of extracting the eluates, and none of the samples was excluded.

Mann Whitney test, also named Wilcoxon Sum U test, was used after the Shapiro-Wilk normality test showed that the data was not normally distributed. Results showed a clear significant difference between the two methods of extraction (Figure 4). Although the two extraction methods used the same kit, QQ small RNA concentrations were significantly higher than the QM concentrations (p-value <0.0001). Aldosaky (2020) got similar results when comparing manual and QIAcube extraction of miRNA with another kit on 275 µl and 100 µl of plasma. One reason for this difference could be the volume of isopropanol used during the extraction process.

Isopropanol was used to bind all RNA, including miRNAs, to the spin column membrane; thus, all the contaminants can be washed away (miRNeasy® Serum/Plasma Advanced kit, 2020). In miRNeasy® Serum/Plasma Advanced kit from Qiagen, Protocol: Purification of Total RNA, Including miRNA, From Serum and Plasma, step seven states, "Add one volume of isopropanol" to the supernatant which is equivalent to "~230 µl for 200 µl serum/plasma". Therefore, QIAcube (Qiagen) set this volume of isopropanol as default for this protocol and cannot be changed. However, the project worked on 100 µl of plasma only, and the isopropanol should be decreased to ~120 µl. The amount used during the QM was around 120 µl, but for the QQ, the volume of isopropanol was not changed and followed as mentioned in the protocol 230 µl. It is possible that the higher volume added by QQ increased the binding of the RNA resulting in higher concentrations in the NS-S RNA eluates. However, more studies are needed to apply 230 µl of isopropanol manually to 100 µl plasma and apply equitable conditions in many QQ and QM samples for a dependable comparison.

Quality control of the extraction methods and purity of total RNA analysis

Another important dimension in assessing the quality of NS-S RNA eluates is the purity and the level of contaminants in each sample. To consider RNA as a pure fragment, purity absorbance 260/280 ratio ($A_{260/280}$) ~2.0 should be achieved (Matlock, 2015). However, higher $A_{260/280}$ do not indicate contamination. Moreover, absorbance ratio 260/230 ($A_{260/230}$) values should be around 2.0–2.2 to ensure RNA purity as a secondary measurement (Matlock, 2015). For QQ and QM extracted total RNA quality results, most of the Ds-11 spectrophotometer (Denovix) measurements were less than the demanded purity level (Appendix C2). Low purity of samples can impact negatively on RT- qPCR efficiency (El-Khoury et al., 2016). El-Khoury et al. (2016) had tested the purity of RNA with three different kits. It was noticed that all of the extracted samples $A_{260/280}$ were less than 1.8. However, the purity increased when the starting material increased by adding MS2 RNA carrier (EL Koury et al.,2016). Moreover, some studies had used miRNeasy® Serum/Plasma Advanced kit (Qiagen) for RNA extraction and showed a low $A_{260/280}$ average. For instance, Norden (2020) had used the kit in the manual extractions of 100 µl of spiked in plasma with miRNA. The mean± SD for $A_{260/280}$ was 1.08 (±1.41). Furthermore, Lindeberg (2020) had used the same kit in the robotic miRNA extraction via QIAcube (Qiagen) of non-spiked 100 µl plasma samples. The mean± SD for $A_{260/280}$ was 0.83 (±0.30), respectively. Thus, from all the mentioned studies, either using manual or robotic RNA extraction, spiked or non-spiked plasma samples, the purity was not high in RNA eluates extracted using miRNeasy® Serum/Plasma Advanced Kit (Qiagen).

Several reasons might be the cause of suboptimal purity. One reason could be the high level of contaminants that naturally occur in plasma, like proteins and inhibitors. (Wright et al.,2020). Another reason could be miRNA binding complexes with lipoproteins in plasma, which reduce the efficiency of isolation kits (Wright et al.,2020). In addition, miRNeasy® Serum/Plasma Advanced kit extracts total RNA and is not specialized with miRNA extraction, increasing the presence of unwanted components in the eluates. Low readings of $A_{260/280}$ might be because of the very low concentrations (less than 10 ng/ µl) of miRNA in the plasma samples (Matlock, 2015). Besides, low readings of $A_{260/230}$ might be due to guanidine thiocyanate in the RPL lysis buffer of the kit and its contamination with the samples (El Khoury et al., 2016)(Appendix B). Additionally, phenol is one of the most common contaminants that decreases nucleic acid's purity (Matlock, 2015). However, this cause can be excluded since the miRNeasy® Serum/Plasma Advanced kit is phenol-free (Qiagen, n.d.)

Outlier and significance test analysis of purity results

The boxplots of QM and QQ purity data showed three outliers in total (Figure 5A). The purity was based on the $A_{260/280}$ ratio as a primary measurement. A purity ratio of more than two is

acceptable (Matlock, 2015). However, one spiked sample QM7(10^6) was a highly deviated outlier where a ratio above six was recorded in QM data. Meanwhile, the sample concentration was not detected (Appendix B2). Purity of samples increases when the starting concentration of RNA increases (El Khoury et al., 2016). Therefore, this outlier indicates that some kind of error occurred during measurement or during laboratory work. Consequently, the outlier was removed (Figure 5B). After the outlier removal, the Mann Whitney test or Wilcoxon Sum U test was used as the Shapiro-Wilk normality test showed that the data was not normally distributed. The results showed a significant difference between QM and QQ total RNA purity (p-value < 0.0001). Total RNA purity in NS-S RNA eluates extracted by QM was significantly higher than the total RNA purity extracted by QQ (Figure 6). The result was not as expected, as QQ eluates were less exposed to contamination by hand since half of the process was carried out by the robotic arm of the machine. However, the machine itself might not be adequately cleaned by ethanol 70% before the runs. Besides, samples prepared for QQ extraction were not on bench work only and samples were moved from one place to another during QIAcube preparation and before processing the machine. This practice might have subjected the samples to a high risk of cross-contamination. Meanwhile, most of the QM NS-S RNA eluates were prepared on a bench with less movement and more precautions.

Hands-on time and turn-around time analysis

Generally, the hands-on time and turn-around time were recorded for six non-spiked plasma samples for RNA extraction after the completion of spiked-in plasma RNA extraction. The reason for that is to have more practical experience with the spiked extractions first and to be familiar with the protocol and the steps before measuring the working time. Time measurements were meant to be close enough as a well-trained laboratory worker who can do QM and QQ extractions in clinical laboratories in the future. It was found that QQ had a shorter hands-on time, but QM had a shorter turn-around time (Table 4). Short hands-on time reduces the risk of cross-contamination since the samples are less touched or handled (Martínez et al., 2006). However, when evaluating for bloodstream pathogen detection like sepsis cases, the turn-around time is critical to consider (Enroth et al., 2019). QM had a shorter turn-around time, but there is a higher chance of human error during manual work with a heavy workload and thus, the process might take longer. In addition, Individual skills and experience may also interfere with the turn-around time from one person to another. QQ had a longer turn-around time than QM, but during the machine operation, there were at least 15 minutes that can be consumed in another laboratory work. For instance, preparing more samples or evaluating results of previously extracted samples (Table 4). The whole argument of whether QM or QQ is better in the extraction process depends on the workflow and routine inside different laboratories (Enroth et al., 2019). However, an apparent assumption can be concluded that QQ could be a suitable option to save effort, time and perform multiple tasks during its operation, especially with a high throughput of samples.

Detection of miRNAs by two-tailed RT-qPCR analysis

Optimization of two-tailed RT-qPCR technology was done, followed by absolute quantification of miRNAs in NS-S RNA eluates. Interestingly, the Two-tailed RT-qPCR method was applied for the first time on NS-S RNA eluates extracted by automation (QIAcube) at the University of Skövde (A.K Pernestig, personal communication, February 22, 2021). However, results were favorable and miRNAs were smoothly detected from NS-S RNA eluates by QIAcube (Qiagen).

Standard curve and absolute quantification analysis

In order to optimize the two-tailed RT-qPCR technology, a standard curve was first analyzed and optimized (Bio-Rad laboratories, 2006). An optimized linear standard curve should have the coefficient of determination $R^2 > 0.980$ or the correlation coefficient $r > |-0.990|$. The results should have high amplification efficiency (E) from 90 % to 105% and a clear consistency across

the replicate reactions (Bio-Rad laboratories, 2006). Although not all the hallmarks had been achieved (Table 5), the optimization of the standard curve was maximized by removing 10^6 and 10^9 copies of synthetic miRNA-210 (Integrated DNA Technologies) (Figure 7). The slope of the standard curve is a measure of the efficiency of the PCR reaction. Ideally, it should be −3.3 for ten folds dilution. Practically, standard curves with a slope between −3.0 and −3.6 are considered acceptable (Overbergh et al., 2017). R^2 measures how well the data fits the standard curve and reflects the standard curve linearity (Life Technologies,2012). The R^2 value did not exceed 0.86 due to inconsistency between the average Cq values between the samples. However, qPCR efficiency is usually the correlated parameter for the robustness and the precision of qPCR assays (Bustin et al.,2009), and this was applicable to the standard curve optimization results as the efficiency was 100%.

Quantification of unknown copies of NS-S miRNAs was done using the optimized standard curve (Table 6A and 6B). Most of the miRNAs in NS-S RNA eluates were successfully detected by the two-tailed RT-qPCR method. All the spiked tested RNA eluates were quantified (Figure 8A). Moreover, the results identified how specific and accurate the two-tailed primers were since the melting curves did not show signs of primer-dimers or secondary amplifications (Bustin et al., 2009) (Appendix D). All the determined copy numbers of miRNAs in NS-S RNA eluates were close to the expected copy numbers with slight variations (Table 6A and 6B). The variation can be due to the individual differences in endogenous miRNAs between donors' plasma since the plasma used in this study was from different adult donors. The Cq values and quantities of miRNA in NS RNA eluates were not detected in three eluates extracted by QM and another three extracted by QQ as mentioned before (data not shown). In addition, samples NS3 and NS10 did not produce amplifications when extracted by QM (Table 6B). However, results were promising, especially that the two-tailed RT-qPCR technology was applied for the first time on endogenous miRNAs extracted from human plasma at the University of Skövde (A.K Pernestig, personal communication, May 30, 2021).

One reason that might explain the failure to detect some samples is the GC content in miRNAs in plasma. Low GC content in the miRNA structure greatly implies its stability (Kim et al., 2012). MiRNA with low GC content can be easily lost during the extraction process, especially when using reagents like guanidine thiocyanate, which exists in miRNeasy® Serum/Plasma Advanced kit (Wright et al.,2020). Another reason could be the multiple freeze/ thaw cycles of the RNA eluates (Yu et al., 2017). Yu et al. (2017) studied RNA degradation with multiple freezing and thawing. RNA degradation occurred with three freeze/ thaw cycles losing 17.75% of its integrity, while with five freeze/ thaw cycles, the RNA lost around 31.29% of its integrity. Furthermore, the plasma used had been frozen at -80°C since 2014, which put speculation that the storage conditions might be changed during this period. In addition to multiple thawing and freezing during the thesis work increases the possibility of miRNA degradation.

The addition of RNA carrier or glycogen before RNA extraction could enhance miRNA recovery and quantification from plasma (Wright et al.,2020). A study by Andreasen et al. (2010) showed an improvement in miRNA quantification after adding MS2 RNA carrier to plasma before RNA extraction, where Cq values were reduced 1–2 Cq cycles compared to RNA extracted without adding the carrier. However, high doses of carrier might mask the actual amount of the extracted RNA as the carrier is counted in the eluates (Moret et al., 2013). Thus, McAlexander et al. (2013) preferred to use glycogen instead of MS2 RNA as a carrier. Their study showed its ability to improve miRNA recovery with some of the extraction kits and without interference with the results of downstream assays.

Results showed that the quantity of miRNAs in NS RNA eluates detected by QQ was higher than the quantity of miRNAs detected by QM (Figure 8B). Meanwhile, the quantity of synthetic miRNA-

210 with endogenous miRNAs in spiked RNA eluates detected by QM was mainly higher than the quantity detected by QQ (Figure 8A). However, the total number of samples quantified from QQ and QM are not trustworthy to conclude which method was better in absolute quantification (Figure 8A and 8B). Moreover, this part of the study aims to optimize the ability of two-tailed RT-qPCR technology to detect and quantify miRNAs as biomarkers from human plasma. Quantification of all the NS-S RNA eluates used in this research was not the primary target due to the project's time constraints. Nevertheless, more research can be done in this area by testing more samples and build a clear perspective. Eventually, this project identified the two-tailed RT-qPCR as a successful method for the detection of natural miRNAs from human plasma. The detection of naturally existing miRNA is considered an outstanding achievement that can be applied to the plasma collected during the Skaraborg sepsis study by Ljungström et al. (2019) (A.K Pernestig, personal communication, May 30, 2021).

Ethical aspects and impact of the research on the society

The study about sepsis had ethical approval (the Regional Ethics Committee in Gothenburg, no. 376–11). All the patients who participated in blood donation had signed informed consent and were aware of the project aim and strategy. However, this thesis work was on plasma extracted from the blood of self-assessed healthy adult donors. The blood was withdrawn from individuals who interestingly wanted to participate in the research with their blood. In this case, no ethical approval was needed.

This project has an imperative impact on the society. It has a powerful effect on infectious disease diagnostics in the future. Biomarkers as miRNAs in the multi-marker panel will help in determining bacterial sepsis from the bloodstream of hospitalized patients or patients admitted to the emergency department as early as possible. This study participates in the implementation of molecular diagnostic techniques in the Swedish healthcare system. The early detection of sepsis will be a revolution in the diagnostics world. It will save millions of lives as clinicians will start with the appropriate treatment immediately without wasting time in trials or treating symptoms rather than the cause of illness. Moreover, complications and the risk of developing MODS will be hugely reduced. The early accurate identification of sepsis will also avoid costly medical expenses and medication waste that hospitals and patients afford due to the long length of hospital stay. Most importantly, there will be a limitation of unnecessary use of antibiotics and a subsequent decline in the rate of antibiotic resistance wide world, which results in a better health and well-being society in line with the united nations foundation 2030 sustainable development goals (United Nations Foundation, n.d.).

Conclusion and Future perspectives

The use of miRNAs as biomarkers for early sepsis diagnosis is auspicious. The extraction of miRNAs manually by miRNeasy® Serum/Plasma Advanced kit (Qiagen) and by QIAcube (Qiagen) was granted in this study. QIAcube (Qiagen) showed that it could compete with manual laboratory work and can be used as a benchmark in the future in clinical laboratories for RNA extractions. Optimization of the two-tailed RT-qPCR (TATAA Biocenter) technology was achieved to a large extent. Detection and quantification of miRNAs by this technology were considered successful from healthy donors' human plasma. The two-tailed primers were able to amplify the target miRNAs without unspecific amplification, which in turn showed the high validity of this new technology.

There were some pitfalls that can be improved in future studies, as the low concentration yield of RNA. This study used one kit for extraction, and this can be improved by comparing more than one kit for QM and QQ extractions and utilizing larger sample sizes. Furthermore, another technique for concentration measurement like Bioanalyzer along with the Qubit can be used (El-

Khoury et al., 2016). Few studies argue about QIAcube (Qiagen) efficacy since it is relatively new automation used for nucleic acid extraction and purification. For instance, A study by Kim et al. (2017) used two automated machines for viral DNA extractions by QIAsymphony RGQ (Qiagen) and QIAcube (Qiagen). The QIAsymphony was more effective in extracting and detecting the viral DNA by qPCR than QIAcube (Kim et al., 2017). Another study compared the extraction of fungal DNA with three protocols, two of them were extracted manually and one extraction was by QIAcube(Qiagen) (Das et al., 2016). The quality of the fungal DNA extracted by QIAcube (Qiagen) was poor compared to the manual extracts (Das et al., 2016). Meanwhile, these studies' results were on DNA extracts, not on RNA extracts which might be considered a biased comparison for the efficacy of the machine. Thus, more studies are needed for RNA extractions by QQ with different kits. This study also showed the ability of two-tailed RT-qPCR technology to detect and quantify miRNAs from healthy donors' plasma. However, it is not yet applied on infected plasma from the human biological material bank (Biobank), which can be the next step in the "Future diagnostics of sepsis" research. Another step afterward is to validate the potential candidate miRNAs (unpublished) that can be used for the multi-marker panel designed for early sepsis diagnosis from sepsis patients (A.K Pernestig, personal communication, June 04, 2021). In the end, the accomplishment of the study makes "Future diagnostics of sepsis" one step closer to the main goal of the project and makes early diagnosis of sepsis possibly come true.

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Appendices

Appendix A: Samples of the thesis project

Table 1. The number of samples planned to be used in miRNA-210 extraction during the thesis work.

Method	The number of spiked plasma samples with miRNA-210 (100µl)	The number of non-spiked plasma samples with miRNA-210 (100 µl)
Manually	12	12
QIAcube	12	12
Total	24 spiked samples	24 non-spiked samples

Table 2. The number of plasma samples spiked in with different copies of miRNA-210 for the thesis work manually and robotically via QIAcube.

Number of miRNA-210 copies/ µl	Number of spiked plasma samples Manually extracted (100 µl)*	Number of spiked plasma samples robotically extracted (100 µl)
10^5	4	4
10^6	4	4
10^7	4	4

*Each spiked-in plasma with the synthetic miRNA-210 sample was around 100 µl.

Appendix B: Quality control values of spiked and non-spiked RNA eluates.

Table 1. Results of small RNA concentrations and total RNA purity absorbance ratio 260/280 for spiked-in plasma samples extracted by QIAcube (Qiagen) using Qubit and Ds-11 spectrophotometer, respectively.

miRNA-210 QIAcube	Small RNA concentration by Qubit (ng/µl)	Total RNA purity $A_{260/280}$	Total RNA purity $A_{260/230}$
QQ1 (10^5)	0.94	0.78	0.11
QQ2 (10^5)	3.10	1.15	0.12
QQ3 (10^5)	0.94	1.19	0.26
QQ4 (10^5)	1.33	1.06	0.17
QQ5 (10^6)	2.34	1.09	0.19
QQ6 (10^6)	2.92	2.19	0.25
QQ7 (10^6)	0.38	1.07	0.10
QQ8 (10^6)	0.52	1.36	0.14
QQ9 (10^7)	0.38	1.14	0.10
QQ10(10^7)	0.72	1.25	0.16
QQ11(10^7)	0.52	1.01	0.12
QQ12(10^7)	0.51	1.54	0.04
Mean± SD	1.22± 0.96	1.24± 0.34	0.15± 0.06

QQ stands for Qiagen QIAcube extraction, and samples are numbered from 1 to 12 with a different number of spiked-in synthetic miRNA-210 copies.

Table 2. Results of small RNA concentrations and total RNA purity absorbance ratio 260/280 in spiked plasma samples extracted manually using Qubit and Ds-11 spectrophotometer, respectively.

miRNA-210 manual	Small RNA concentration by Qubit (ng/µl)	Total RNA purity $A_{260/280}$	Total RNA purity $A_{260/230}$
QM1 (10^5)	1.51	1.43	0.13
QM2 (10^5)	1.05	2.11	0.15
QM3 (10^5)	1.58	1.72	0.05
QM4 (10^5)	1.21	1.16	0.07
QM5 (10^6)	Out of range	1.48	0.48
QM6 (10^6)	Out of range	1.47	0.11
QM7 (10^6)	Out of range	**6.86**	0.21
QM8 (10^6)	Out of range	1.76	0.04
QM9 (10^7)	Out of range	1.15	0.08
QM10(10^7)	Out of range	2.73	0.17
QM11(10^7)	Out of range	1.82	0.07
QM12(10^7)	Out of range	2.73	0.03

Mean± SD	0.41±0.66	2.20± 1.56	0.13± 0.12

Table 3. Results of small RNA concentrations and total RNA purity absorbance ratio 260/280 in non-spiked plasma samples extracted manually using Qubit and Ds-11 spectrophotometer, respectively.

Non-spiked plasma manual	Small RNA concentration by Qubit (ng/µl)	Total RNA purity $A_{260/280}$	Total RNA purity $A_{260/230}$
NS QM1	Out of range	0.87	0.84
NS QM2	Out of range	0.94	1.11
NS QM3	Out of range	1.43	4.22
NS QM4	Out of range	1.85	0.49
NS QM5	Out of range	1.59	4.58
NS QM6	Out of range	1.22	2.32
NS QM7	Out of range	1.14	2.08
NS QM8	Out of range	1.58	5.29
NS QM9	Out of range	1.56	0.75
NS QM10	Out of range	1.49	1.69
NS QM11	Out of range	1.96	2.53
NS QM12	0.710	1.48	3.49
Mean± SD	--	1.43± 0.33	2.45± 1.61

Table 4. Results of small RNA concentrations and total RNA purity absorbance ratio 260/280 in non-spiked plasma samples extracted by QIAcube (Qiagen) using Qubit and Ds-11 spectrophotometer, respectively.

Non-spiked plasma QIAcube	Small RNA concentration by Qubit (ng/µl)	Total RNA purity $A_{260/280}$	Total RNA purity $A_{260/230}$
NS QQ1	0.28	0.86	0.11
NS QQ2	0.15	0.99	0.13
NS QQ3	Out of range	0.76	0.05
NS QQ4	Out of range	0.82	0.08
NS QQ5	Out of range	0.54	0.05
NS QQ6	Out of range	0.68	0.08
NS QQ7	1.14	0.71	0.08
NS QQ8	0.39	1.09	0.12
NS QQ9	1.20	0.77	0.09
NS QQ10	1.26	0.65	0.09
NS QQ11	0.41	0.68	0.09
NS QQ12	0.93	0.63	0.09
Mean± SD	0.48±0.51	1.24± 0.16	0.09± 0.03

Appendix C: Quality control values of all RNA eluates based on extraction methods.

Table 1. Results of all small RNA concentrations extracted manually and by QIAcube before outlier removal. The number of samples is 24 in each group (n=24).

Sample number	Small RNA concentration extracted by QIAcube (QQ) (ng/µl)	Small RNA concentration extracted Manually (QM) (ng/µl)
1	0.94	1.51
2	3.10	1.05
3	0.94	1.58
4	1.33	1.21
5	2.34	NA
6	2.92	NA
7	0.38	NA
8	0.53	NA
9	0.38	NA
10	0.72	NA
11	0.52	NA
12	0.51	NA
13	0.28	NA
14	0.15	NA
15	NA	NA
16	NA	NA
17	NA	NA
18	NA	NA
19	1.14	NA
20	0.39	NA
21	1.20	NA
22	1.26	NA
23	0.41	NA
24	0.94	0.71
Mean± SD	0.25± 0.52	0.85±0.86

Table 2. Results of total RNA purity measurement extracted manually and by QIAcube before outlier removal. The number of samples is 24 in each group (n=24).

Sample number	Total RNA $A_{260/280}$ extracted by QIAcube (QQ)	Total RNA $A_{260/280}$ extracted extracted Manually (QM
1	0.78	1.43
2	1.15	2.11
3	1.19	1.72
4	1.06	1.16
5	1.09	1.48
6	2.19	1.47
7	1.07	**6.86**
8	1.36	1.76
9	1.14	1.15
10	1.25	2.73
11	1.01	1.82
12	1.54	2.73

13	0.86	0.87
14	0.99	0.94
15	0.76	1.43
16	0.82	1.85
17	0.54	1.58
18	0.68	1.22
19	0.71	1.14
20	1.08	1.58
21	0.77	1.56
22	0.65	1.49
23	0.68	1.96
24	0.63	1.48
Mean± SD	1.00± 0.36	1.81± 1.17

Appendix D: Melting curves of spiked and non-spiked RNA eluates

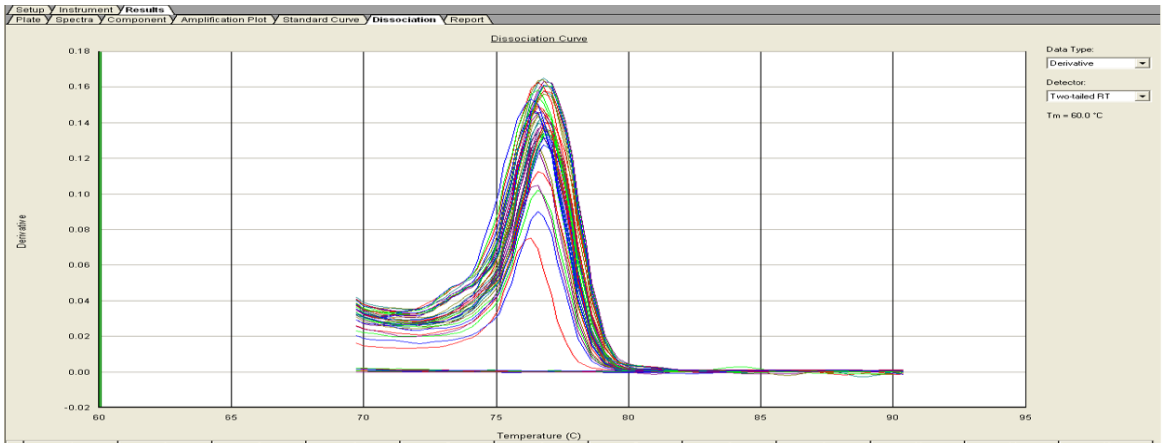

Figure 1. A Melting curve shows one peak for spiked-in plasma samples with miRNA-210 amplification and undetected negative controls.

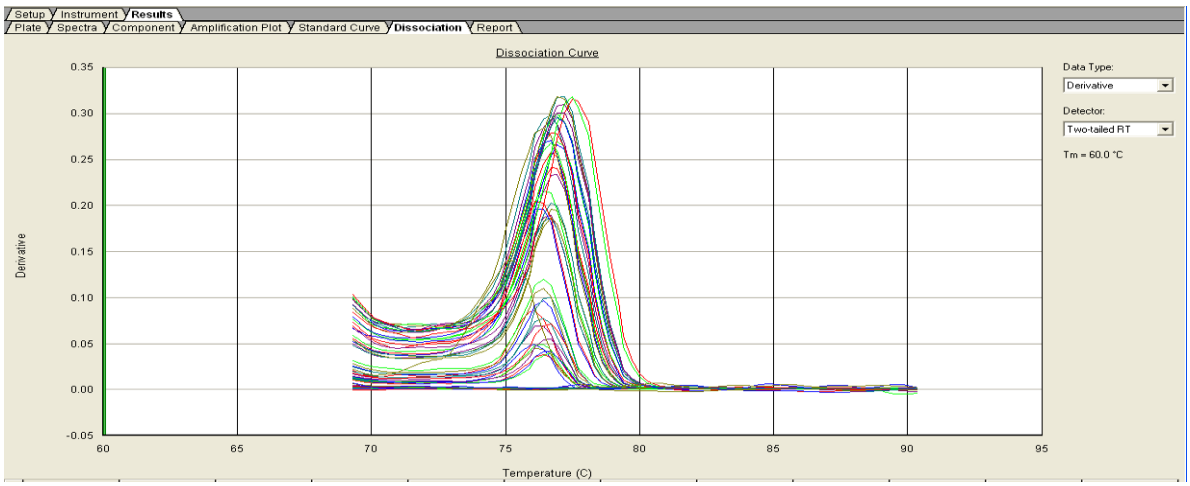

Figure 2. A melting curve shows a single peak amplification of non-spiked plasma samples and undetected negative controls.

Appendix E: Synthetic miRNA-210 sequence and two-tailed RT-qPCR primers

miRNA target	miRNA sequence	RT primer sequence	qPCR primers (forward/reverse)
miR-210	AGCCCCUGCCCACCGCACACUG	GGCTGTAACCATATTTGTTACAGTGAGTAACACGATAGATGCCAGTGTGC	GGCTGTAACCATATTTGTTAC
			AGCCCCTGCCCAC

Figure 1. Synthetic miRNA-210 from Integrated life technologies sequence with RT-primer sequence and qPCR forward and reverse primers' sequences.