Degree project

UNIVERSITY OF SKÖVDE

Comparison of manual and semi-automatic RNA extraction methods using two-tailed RT-qPCR for absolute quantification as part of the sepsis diagnosis research

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Student: Elia Callado Prat
d22elica@student.his.se

Supervisor: Anna-Karin Pernestig
anna-karin.pernestig@his.se
Johan Nordén
johan.norden@his.se

Examiner: Maria Algerin
maria.algerin@his.se

School of Bioscience
University of Skövde
Box 408
541 28 Skövde

Abstract

Nowadays, sepsis has become a major healthcare problem. Its variance of symptoms and the lack of time to act makes it greatly difficult to treat. An early diagnosis using biomarkers, particularly miRNA, could potentially increase the patient's prognosis as well as reduce the use of antibiotics for the treatment. The lack of method optimization for miRNA extraction and quantification calls for investigation prior to the construction of a multi-biomarker panel for sepsis diagnosis. The aim of this project was to examine and compare manual and semi-automatic extraction methodologies through the small RNA quantity and RNA quality, as well as test the detection and quantification abilities of the novel technique, two-tailed RT-qPCR. 30 extractions have been performed, their extracted elutions have been subjected to quality and quantity control and detection and absolute quantification through the two-tailed RT-qPCR. The results show no significant differences between the quantity and quality of the RNA extracted using both methods. Time management, on the contrary, reported significant differences between the two methods. On the other hand, the two-tailed RT-qPCR successfully amplified the miRNA candidate from as little as 100 μL of healthy plasma. The absolute quantification showed the miRNA candidate's low concentration in plasma. Moreover, the qPCR efficiency was irregular during the project which may alert of contamination or unspecific primers. However, the melt curve showed a single amplicon which suggests great specificity. The detection and quantification of the miRNA candidate have been successful, though further investigation is recommended.

Abbreviations

BSI – Bloodstream infections

cDNA – Complementary desoxyribonucleic acid

Cq – Quantitation cycle

CRP – C-reactive protein

DNA – Deoxyribonucleic acid

HOT – Hands-on time

IQR – Interquartile range

LOD – Limit of detection

LOQ – Limits of quantification

MALDI-TOF – Matrix-assisted laser desorption/ionization time-of-flight mass spectrometry

miRNA – microRNA

NEWS – National Early Warning Score

NTC – Non-template control

PCR – Polymerase chain reaction

PCT – Procalcitonin

qPCR – Quantitative polymerase chain reaction

RCF – Relative centrifugal force

RNA – Ribonucleic acid

RT – reverse transcription

SD – Standard deviation

SOFA – Sequential Organ Failure Assessment

TAT – Turnaround time

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Introduction

The condition of sepsis

Throughout the years, a large number of specialists have tried to define the syndrome of sepsis. A total of three consensus conferences have been celebrated for that purpose, the last and most recent took place in 2014 (Verdonk et al., 2017). According to this consensus, sepsis is defined as a life-threatening organ dysfunction caused by a dysregulated host response to infection. Additionally, there is the sepsis shock, a subset of the sepsis syndrome that occurs when the patient has been treated but despite this, there is a persistent condition of hypotension. It increases the chances of death (Esposito et al., 2017).

The reason behind the numerous adjustments in the definition is the complexity and variability of the sepsis pathophysiology. There are some characteristics which are fairly common among sepsis patients, such as changes in body temperature and coagulation of blood (Hotchkiss & Karl, 2003; Schortgen, 2012; Levi & van der Poll, 2017). However, there are many others that differ and therefore are not conclusive.

The origin of sepsis is the infection that affects the body. Though the source of the infection has a wide range as well. The infection may be caused by bacteria, fungi, parasites, etc. Even viruses have been known to cause sepsis (Lin et al., 2018). The disparity of the microorganism that causes the infection only contributes to the difficulty of diagnosing this syndrome. Nevertheless, it is true that the most common causative of sepsis is gram-positive bacteria followed by gram-negative bacteria (Martin, 2012).

There are certain factors that can increase the possibility of a patient getting sepsis, the main ones being age and comorbidities (De La Rica et al., 2016). The age presents a bimodal distribution, the risk increases around children, decreases as they grow, and then increases again as they reach elderhood. Comorbidities have more to do with the fact that the body is already fighting and weakened, as well as possibly having immunosuppression, which would make the immune system unable to fight the infection. Other minor factors are sex, women suffer slightly less from this condition, and season, since in winter there are more pulmonary infections (Esposito et al., 2017). Additional possible risk factors have proven to be cirrhosis (Foreman et al., 2003) and alcohol dependence (O'Brien et al., 2007) among others.

Once sepsis has begun to act, the responses of the patient as well as the severity of it are unique in every case. Organ dysfunction is usually paired with a deficient reaction of the immune system. Moreover, sepsis advances as either the patient cannot hold against the primary infection or because of overdrive from the immune system. The low efficiency of the immune system has been regarded as a component of late death in patients with sepsis for a long time now (Meakins et al., 1977).

Sepsis affects the coagulation of blood, increasing the procoagulant agents and decreasing the anticoagulant. The alteration of the signalling pathways in the sepsis condition ultimately induces tissue injury and organ failure (Russel, 2006).

The prognosis of deathly sepsis depends on a number of factors. First of all, it depends on the patient's risk factors that may already be present and have the ability to worsen the prognosis, as mentioned before. In the second place, the characteristics of the infection that originated the sepsis, for example, high virulence microorganisms and bloodstream infections are related to unfavourable outcomes. Third is the intensity of the patients' response to the infection. Lastly, there is a positive correlation between the number of organs failing and mortality (Gustot,

2011).

A study recently performed by Rudd et al. (2020) confirms that sepsis is still a considerable cause of mortality globally. During the year 2017, there were approximately 48.9 million cases of sepsis worldwide, of which 11 million could not overcome the syndrome complications and passed away. This is the first study which has examined the incidence of sepsis globally, what has been made clear is that the global burden of sepsis is larger than previously appreciated and is in need of urgent attention.

Current diagnostics of sepsis

Notwithstanding, it has come to attention that an early diagnosis of sepsis could both improve the outcome of the patients and minimise the costs of the treatment. Since the sooner the doctors have the facts, they can organise a specific treatment and improve the patients' prognosis.

Up until now, the diagnosis of sepsis has revolved mainly around blood culture methods. In a standard manner, every patient who has indications of infection should have control of their vital signs as well as a white blood cell count as soon as possible. Swiftly following those measures, there is the search for sepsis-induced organs (O'Brien et al., 2007). Though it may vary depending on the health institution.

Medical history, clinical symptoms, vital parameters and routine biomarkers are used to diagnose sepsis along with the detection of microorganisms in the blood or other parts of the body using blood cultures. The use of blood cultures is crucial for a correctly adapted antibiotic therapy. However, there are factors that may taint the results of the cultures such as antibiotics being administered before taking the blood samples (Esposito et al., 2017).

Nowadays, the "gold standard" for BSI (bloodstream infections) diagnostics is based on blood cultures, which allow the recognition of viable microorganisms in the blood, followed by subculture in order to identify the microorganism grown in agar plate (Enroth et al., 2019).

For every suspected infection focus on the body there should be samples gathered. First, the microorganism must grow. The guidelines propose collecting 40 to 60 mL per focus and dividing it equally between an aerobic bottle and an anaerobic bottle (Mancini et al., 2010). Once they have grown, they need to be identified. The techniques to detect the microorganisms vary widely, from amplification and hybridization to mass-spectrophotometry based (such as MALDI-TOF).

A recent study performed by Ljungström et al. (2019) reported that approximately 38% of sepsis patients have negative blood cultures, therefore if the microorganism responsible for the origin of the infection is recognized it could mean a great help in deciding the medication and consequently decreasing the possibility of resistant organisms.

The blood cultures may provide results from 48 to 96 hours (Esposito et al., 2017), and afterwards, there is the identification period which adds to the already lost time. This is a major problem since the syndrome of sepsis acts fast and the patient has approximately 4 hours before death in the worst of cases.

The Sequential Organ Failure Assessment (SOFA) score was created with the purpose of estimating the acute morbidity of critical illness at a population level; it has been broadly accepted as an instrument for medical care. Since its development, the original goal has expanded and, following the changes in the new definition of sepsis, is now a key criterion for the diagnosis of sepsis on an individual level (Lambden et al., 2019).

Organ dysfunction can be detected by the change in total SOFA score, a minimum of two points ensuing the infection (which increases the mortality by approximately 10%). In patients who had never suffered from organ dysfunction, it is assumed a SOFA score of zero (Verdonk et al., 2017). Despite SOFA being the most used system, there are others such as the National Early Warning Score (NEWS) which is the one used in Sweden with a sensitivity of 58% and specificity of 81% for the diagnosis of sepsis (Oduncu et al., 2021).

Biomarkers as a diagnostic tool

Even with all these resources, the diagnosis of sepsis is uncertain or takes too much time. That is why in the last two decades, researchers have begun to study biomarkers. Biomarkers are measurable indicators of a biological state or condition (Benz et al., 2016). As time goes by, more and more physicians are using biomarkers to diagnose sepsis and predict the outcome.

The use of biomarkers in the process of sepsis diagnosis would mean a great advantage since they would be able to discern between the presence or lack of sepsis, as well as the severity of it. They are also able to differentiate between the causative microorganism and whether it is a local infection or a systematic one (Marshall & Reinhart, 2009).

A molecule that wants to be used as a biomarker needs to fulfil certain conditions. The molecule should be of easy access and obtainable using only non-invasive, cheap and fast methods. It also needs to have a certain specificity to the injury/pathology the investigators want to use it for (Dumache et al., 2015).

Currently, the three more used biomarkers in sepsis diagnosis are procalcitonin (PCT), C-reactive protein (CRP) and lactate (Diaconu et al., 2015). It has been proved that by using PCT for the diagnosis, the length of antimicrobial treatment could be lowered by 2 to 3 days (Westwood et al., 2015), it also has the highest specificity to sepsis so far (despite being still under 90%)(Pierrakos & Vincent, 2010). PCT high levels are highly related to the sepsis condition and are, consequently, listed by the Sepsis Guidelines as diagnostic biomarkers (Dellinger et al., 2013). It can also be useful to exclude the possibility of sepsis rather than diagnose it, PCT has a high negative predictive value of 99% (Liaudat et al., 2001).

CRP is typically applicable for the detection of inflammatory or infectious activity, however, the specificity in sepsis is lacking (Esposito et al., 2017). Nevertheless, its high serum levels are useful to determine the gravity of the infection (Provoa et al., 2005) and its low levels after a treatment is applied may indicate if said treatment worked (Schmit & Vincent, 2008).

Serum lactate has been used to guide sepsis therapy in the last years, it is also really useful for monitoring organ dysfunction (Rivers et al., 2001).

Another group that has been highly studied are the cytokines. They are very present in the pathophysiology of sepsis and appear relatively fast after sepsis is manifested (Pinsky et al., 1993). However, their blood concentration takes an erratic pace and does not match the progress of the condition (Wu et al., 2009).

Although, up until now, there has not been any biomarker with enough specificity to differentiate between sepsis and other inflammatory afflictions (Esposito et al., 2017). More recently a novel biomarker was introduced: microRNA. MicroRNA (miRNA) form an RNA category of small (20-24 nucleotides) molecules that do not code for proteins, instead, they modulate gene expression. It is calculated that miRNA regulates up to 60% of all protein-coding genes (Krol et al., 2010).

miRNA has been detected in blood and has been proven to be useful as a biomarker (Chen et al.,

2008). Particularly, circulating miRNA has an elevated resilience in conditions that would break most other RNA, such as extreme temperatures or changes in pH. Moreover, they have more advantages in front of regular protein-based markers: they are moderately small, do not have post-translation modification and have a simple chemical structure (Benz et al., 2016). miRNAs are also closely involved in the immunological process in pathological disorders (O'Connell et al., 2012).

There have been multiple deregulated miRNAs described in the blood of patients with infectious and inflammatory diseases during the last few years (Benz et al., 2016). This leads to the idea of using circulating miRNA as possible biomarkers for critical illnesses and sepsis (Correia et al., 2017). Although caution is recommended given how the place where the miRNA is extracted is of great importance to the result, since each biofluid may be enriched with different sets of miRNAs. (Weber et al., 2010).

Challenges with the extraction and quantification methodology

A recurring problem with using circulating miRNA as a biomarker is the discordance between investigations that may be ascribed to the absence of data standardisation and other technical difficulties (Moldovan et al., 2014). This discrepancy may lead to erroneous discoveries regarding biomarkers. Meticulous supervision of the quality of the samples and the technical workflow is consequently essential.

In order to achieve the successful implementation of all of the recent progress in miRNA research, a number of challenges need to be addressed. And of these, first and foremost, is the accuracy and reproducibility of the miRNA quantification measurements. Currently, there is not much consensus on which methodologies are adequate for sample collection, miRNA extraction, quantification and data analysis. Moreover, in the field of clinics, reproducibility and repeatability are essential for the successful implementation of miRNA as biomarkers (Witwer, 2015). Thus, the development of robust tools to enable the standardisation of miRNA data is required.

An easy and effective course of action to detect technical variation would be to perform a spike-in prior to the extraction of the miRNA (Buschmann et al., 2016). This way is simple to detect anomalous results from samples that should either be re-examined or eliminated.

An important factor that is often overlooked is the starting volume of the sample that is going to be used for quantification (El-Khoury et al., 2016). Given the miRNAs' low levels in the biofluids, it might be tempting to increase the initial volume, however, the more the starting volume increases so does the risk of carryover of contaminating molecules and saturation of purification (Brunet-Vega et al., 2015). Another factor to take into account is that the starting volume of the sample is different depending on the body fluid as well as the species of animal from whom the biofluid comes from (Androvic et al., 2019).

Multiple commercial kits have emerged with the intention of isolating RNA, these are usually preferred to the traditional RNA isolation protocols since the kits provide a quicker and usually easier method for isolation. Albeit it is worth mentioning the fact that miRNAs are usually associated with protein complexes and RNA-binding proteins, which increases the complication of miRNA extraction and quantification (Wright et al., 2020).

One example of a comparison of different extraction kits is in the article of Sriram et al. (2021). Four different miRNA extraction kits were compared. Out of all of them, the miRneasy Serum/Plasma Advanced kit was reported to be the best option. It has the highest recovery score, not to mention that it ensures higher quality and quantity of miRNA in comparison to the other kits studied. Moreover, it shows maximal miRNA upon elution. The fact that it is simple to

use, and it is not time-consuming only adds to its benefits. Not only in this article is this kit recommended but also other articles (Androvic et al., 2019; Garcia-Elias et al., 2017) considered it the best option.

There are many biofluids from which the miRNA can be extracted, yet plasma is one of the trickiest ones. In the case of isolation from plasma, one worrying difficulty is the contamination from miRNA derived from lysed blood cells, especially hemolyzed erythrocytes (Kirschner et al., 2011). To check if this complication has taken place, the habitual method is to measure the absorption at 414 nm, 540 nm and 578 nm (which match with the absorption peaks for free oxyhemoglobin) (Kirschner et al., 2013).

As for the quantification of miRNA, the three most commonly utilised methods are microarrays, next-generation sequencing (RNA-seq) and reverse transcription quantitative PCR (RT qPCR) (Androvic et al., 2017). All of these techniques have their own strengths and weaknesses. According to Garcias-Elias et al. (2017), the spectrophotometers methods overestimate the miRNA levels since they also detect other contaminants molecules (such as other nucleotides or proteins), while choosing the Qubit 2.0 fluorometer as the most specific quantification method for miRNA. Even though the sequencing of the obtained miRNA showed that only 58% of the RNA collected is true miRNA. Qubit has a restriction, however, since this method is prone to detect all kinds of small RNA.

Apart from these, new methods are emerging, such as the two-tailed RT qPCR. The two-tailed RT qPCR is a novel RT qPCR-based technique. It revolves around sequence-specific RT primers which have a new design that allows the RT primer to hybridise into two regions of the miRNA target with separate complementary parts named hemiprobes. This technique provides benefits in front of the already existing ones, namely high sensitivity, high differentiation between similar miRNA and the ability to quantify isomiR (which would thoroughly reflect the real miRNA content in the sample). Moreover, it allows quick testing with a total analysis time of less than 2,5 hours (Androvic et al., 2017).

Thesis project

The study of biomarkers as a diagnostic tool has not only been intended for sepsis, yet sepsis is the one condition for which the most effort and research have been used. Multiple studies have assessed the ability of up to 180 biomarkers to perceive infections, inflammation and sepsis (Benz et al., 2016).

There is no single biomarker ideal for the sepsis diagnosis, of course taking into account the irregularity of sepsis it was to be expected that a sole biomarker would not cover all of the variability displayed. That is the reason a kit of multiple biomarkers started to be considered (Ljungström et al., 2017). The kits would be formed with five to fifteen different biomarkers, which together could inform efficiently if the patient suffers from sepsis. Now the challenge lies with choosing the right combination of biomarkers that can diagnose sepsis early, efficiently and specifically. The creation of this kit is the ultimate goal, but in this project, the main focus will remain on a different part of the process. Specifically in the detection of miRNA candidates using the methods proposed.

Knowing that miRNA presents a promising diagnostic value in regard to sepsis, the development and assessment of methods for the extraction and quantification of miRNAs are required in order to prove that they really supply consistent and reliable results. The aim of this project was to examine and compare manual and semi-automatic extraction methodologies through the small RNA quantity and RNA quality, as well as test the detection and quantification abilities of the novel technique, two-tailed RT-qPCR. Thus, creating a standardisation procedure that can be easily reproduced for measuring miRNAs in human plasma. The overall objective is defined as

the analysis of the miRNA candidate, miR-seps 5, extracted from healthy plasma to become part of a future multiplex kit to diagnose sepsis.

The project has investigated two principal research questions. In the first place, can miR-seps 5, the miRNA candidate, be detected and quantified accurately from human plasma using two-tailed RT-qPCR? miRNAs properties differ widely and consequently so does its behaviour (Redshaw et al., 2013), which results in divergent outcomes during extraction and quantification. Thus, despite past favourable results in other studies, there is no assurance that the technique will produce the same developments with the miRNA candidate. Nevertheless, articles (Androvic et al., 2017; Voss et al., 2021) have provided auspicious results that lead to believe that the two-tailed RT-qPCR can yield highly sensitive, specific and reproducible results. Consequently, the hypothesis was that it would produce akin results in the thesis project.

In the second place, is it possible to obtain higher quality and quantity of miRNA with the semi-automated extraction using the QIAcube (QIAGEN) compared to manual extraction, where the MiRneasy Serum/Plasma Advanced kit (QIAGEN) will be used? Using the QIAcube to do the extraction may reduce the repercussions of handling bias, hence providing more congruent results than the manual extraction would (Sharma et al., 2022). Even so, there is no certainty that the parameters established in the semi-automatic technique will be adequate for the miRNA candidates. To see first-hand the benefits and disadvantages of these techniques, both extractions methodologies has been used and quantified using two-tailed RT-qPCR to examine if there was a significant difference between them. The hypothesis, in this case, was that the semi-automatic extractions would be of higher condition than the ones provided by the manual procedure. Additionally, there is also the amount of time invested in each of the methodologies to consider. For that reason, the hands-on time, HOT, and the turn-around time, TAT, have been measured.

These two questions are valuable for the ongoing research of “Future diagnostics of sepsis”, carried out by the School of Bioscience of the University of Skövde, Sweden. As this subject is still open to investigation, the miRNA candidates will be referred to using a pseudonym.

Material and methods

Ethical approval

The ongoing research of “Future diagnostics of sepsis” had ethical approval for this study (the Regional Ethics Committee in Gothenburg, no. 376–11). The patients with suspected sepsis who contributed with their biofluids to the biobank signed an informed consent.

There was also a parallel study performed, which was not included in the ethical approval since it was not needed. Regardless of that, they did need to inform the healthy blood donors of what they wanted to do with their blood and have their oral consent. These samples are the ones that has been used in this project.

Obtention of the plasma and its storage

The blood used in this study was collected from the middle part of the arm of healthy blood donors. It has been drawn from a Venous source as whole blood. The samples were collected initially in the year 2014 using a six mL K2E K2EDTA Vacuette tube (Greiner bio-one) and then centrifuged at room temperature for 10 minutes, with a speed of 2000 relative centrifugal force (RCF) in order to isolate plasma from other blood components. Citrate was used in order to avoid coagulation. Each prepared plasma fluid was later transferred and divided into smaller

tubes, with 300 µL volume in each, and frozen in a - 80ºC freezer, in the University of Skövde laboratory.

Study outline

In the first place, there has been the process of optimization. The optimization was composed of two parts. The first one would be formed by two plasma samples (from the same blood donor), each sample was split into three (100 µL per tube). Each tube then was spiked with different amounts of synthetic miR-210 (Integrated DNA Technologies): 10^5, 10^6 and 10^7 copies/µL. One of the plasma samples was processed manually (MiRneasy Serum/Plasma Advanced kit) and the other semi-automatically (with the QIAcube). Once all six of the samples have been subjected to quality and quantity control and two-tailed RT-qPCR, the process advanced to part two.

After going through the results of part one, the lowest spike-in concentration with which there has been amplification during the two-tailed RT-qPCR was selected. For this part, there have been three new plasma samples (from the same blood donor), and for each of these two extractions using the synthetic miR-210 amount of copies previously mentioned. One by manual means and the other using the QIAcube (QIAGEN), though all of them have been spiked with the previously chosen concentration (Figure 1).

After the optimization, there have been some (n=12) spiked extractions using synthetic candidate miR-seps 5 (Integrated DNA Technologies)to act as a positive control with the same spike-in amounts as the optimization. Once this was completed, the non-spiked plasma stage could start (Figure 1). In this part of the project, there was no added synthetic miRNA, it has only consisted of the endogenous miRNA already found in plasma. A total of 30 extractions from non-spiked plasma have been conducted, 15 done by manual means, and the remaining 15 done using the QIAcube (QIAGEN). All the non-spiked extractions have been done with plasma from the same healthy blood donors (different from the one used in the optimization and positive controls).

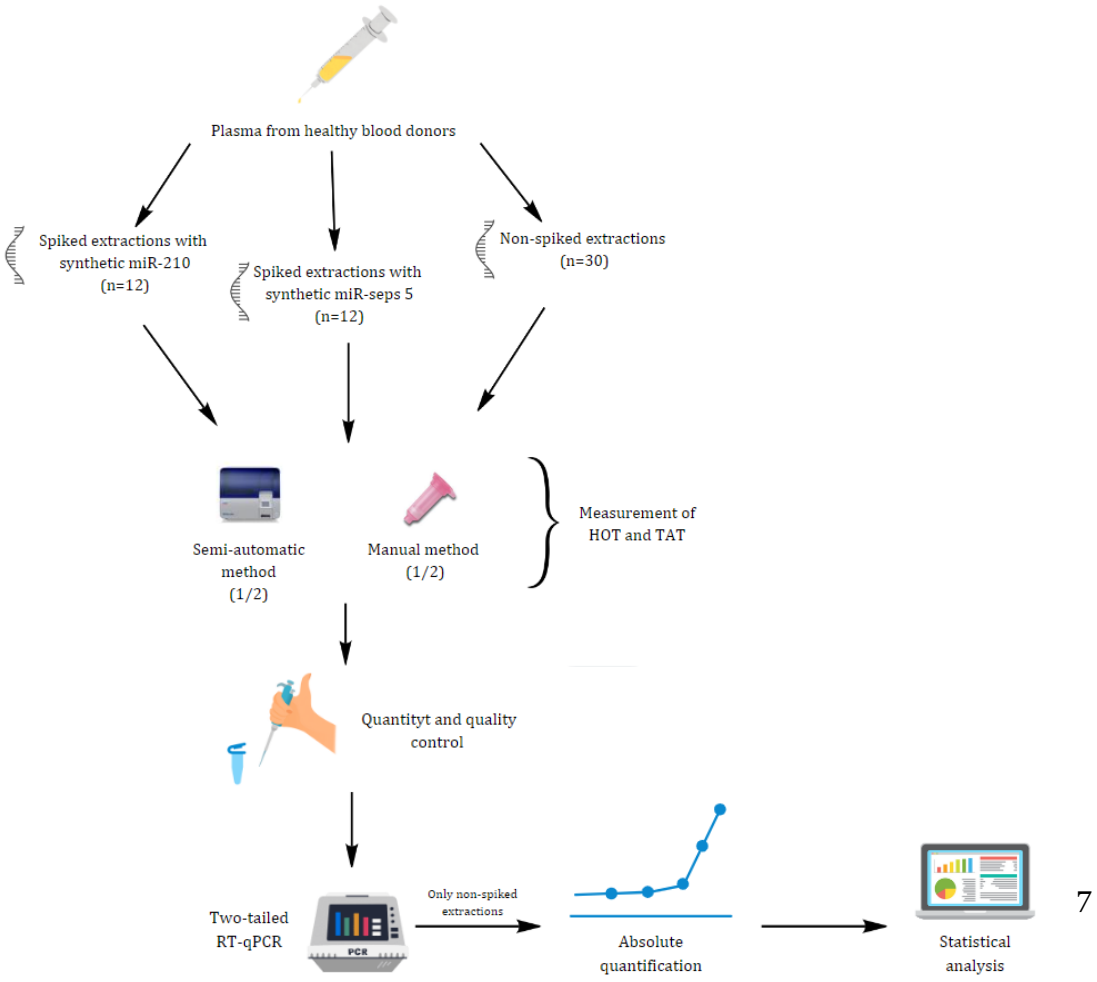

Figure 1. Schematics of the experimental design for the whole project, the results displayed in this report are the ones performed in the non-spiked stage. The division 1/2 in the extractions methods represents that in every stage (optimization, positive controls and non-spiked extractions) half of the extractions were done using the manual method consisting of the MiRneasy Serum/Plasma Advanced kit (QIAGEN) while the other half were performed with the semi-automatic using the QIAcube (QIAGEN). HOT stands for hands-on time and TAT for turn-around time, both of which have been measured to estimate time management. The quantity and quality control has assessed has been performed using the Qubit microRNA Assay (Thermo Fisher Scientific) and the nanodrop (Thermo Fisher Scientific). The absolute quantification has been obtained through the two-tailed RT-qPCR. Once everything was done, the non-spiked extractions were subjected to absolute quantification and statistical analysis.

Extraction of the small RNA and time management

The manual extraction has been done following the steps of the MiRneasy Serum/Plasma Advanced Kit protocol (QIAGEN, 2021). Albeit the recommended starting volume according to this kit is of 200 µL, it has been replaced by a starting volume of 100 µL. With this modification, the Buffers RPP and RPL also needed to be downscaled accordingly. Instead of the original 60 µL of the Buffer RPL, 30 µL has been used, and as opposed to the original 20 µL of the Buffer RPP only 10 µL has been enough.

During the manual extraction, 115 µL of the supernatant obtained from centrifugation was pipetted and blended with 115 µL of isopropanol. Following this, 230 µL of the mixture was pipetted to proceed with the MiRneasy protocol (QIAGEN, 2021). In order to perform the spike-in, synthetic miRNA (Integrated DNA Technologies) has been used. During the first phase of optimization, miR-210 spike-in were done using three different amounts of copies, as previously mentioned. The established concentrations were obtained through ten-fold serial dilution of an original solution of synthetic miR-210 (Integrated DNA Technologies) 10^{13} copies/µL. During the second phase, the spike-in were obtained with the same original solution and consequential dilutions, however, only the concentration selected was used. For the candidate spiked controls, the spike-ins were obtained through serial ten-fold dilution of an original solution of 10^{13} copies/µL of synthetic miR-seps 5 (Integrated DNA Technologies), the first six extractions using 10^5, 10^6 and 10^7 copies/µL and the last six only using 10^5 copies/µL as the smallest amount of copies with amplification.

The semi-automatic extraction procedure has been composed of part of the manual protocol followed by the use of the QIAcube (QIAGEN). Steps one to five from the MiRneasy protocol (QIAGEN, 2021) have been repeated. A point to take into account is that the QIAcube is programmed to extract the miRNA from a starting volume of 200 µL, however, using the minimum starting volume (100 µL) is a key part of the project. Therefore, adding 100 µL of RNAse-free water during step two in the protocol was enough to correct this. Once the necessary steps from the protocol were completed, 230 µL of supernatant was placed in the QIAcube following the loading chart (QIAGEN, 2018) and initiated the procedure. The spike-in process has been done with the same number of copies as the manual one and was also added after the third step in the MiRneasy protocol.

In addition, two more parameters were observed. First of all, there was the Hands-on time (HOT) which is the time measured when manual handwork was performed (from this is excluded the fixed time such as centrifugation or incubation). Second, the Turnaround time (TAT) is the time needed to accomplish the whole extraction task. Both the starting time and the finishing time of the procedures were written down for posterior calculation of the times. TAT includes from the start to the end of the extraction, while HOT is TAT minus the fixed time. In the manual extraction, the fixed time was of 24 minutes and 15 seconds while in the semi-automatic extraction it was variable. Measuring this also helped in the comparison between the manual and robotic extractions.

Quantity and quality control

Following the extraction, the results provided by both methods were compared in terms of small RNA quantity and RNA quality. The extracted elution was placed into the Qubit 4.0 Fluorometer (Thermo Fisher Scientific) using the reagents from the Qubit® MicroRNA Assay (Thermo Fisher Scientific). The assay has been performed as suggested in the protocol (Thermo Fisher Scientific, 2015), with two standards performed before introducing the extracted eluted. For this, three µL of elution was combined with 197 µL of the working solution prepared according to the protocol (Thermo Fisher Scientific, 2015). With this, the quantity of small RNA in the elution was obtained. The inferior limit in the Qubit® microRNA assay (Thermo Fisher Scientific) is 0.5 ng (Thermo Fisher Scientific, 2022), the extractions with value below that value have been replaced with a constant through the LOQ/$\sqrt{2}$ equation (Bergstrand & Karlsson, 2009).

Following this measurement, the DS-11 Series Spectrophotometer (DeNovix) was used for the evaluation of the quality. Using the same elutions from the extractions, one µL of the elution for analysis was placed in the machine. Before putting the sample, there needed to be one µL of RNAse-free water to draw a blank and make sure the results were as exact as possible.

Two-tailed RT-qPCR

After performing the quantity and quality control, the extracted samples were conserved in the freezer at -20ºC until further use. The GrandsScript cDNA FreePrime Kit (TATAA Biocenter) was used to perform the reverse transcription (RT) reaction in order to obtain cDNA. For this reaction, the two-tailed primer for miR-210 (optimization) and miR-seps 5 (candidate extractions) was used. On the other hand, the quantification of the cDNA was done using the SYBR GrandMaster Mix (TATAA Biocenter). In this case, the forward and reverse primers for miR-210 and miR-seps 5 were the ones in use. For each qPCR reaction, three non-template controls (NTC), composed solely by Master Mix, were performed to make sure that no contamination had ensued. Both protocols have been slightly altered in this project from the original versions, the exact steps can be consulted in Appendix 1.

After the qPCR program, performed with an AriaMx Real-time PCR System (Agilent Technologies), was finished the results were retrieved from the machine using a pen drive. The values of the results have been observed with the AriaMx Version 2.0 Software. This software was also able to classify the extracted elutions depending on their well type, whether non-template control (NTC), standard or others, as well as their starting amount in the case of the standard curve.

Absolute quantification

In order to quantify the miR-seps 5 levels in the non-spiked extractions, a standard curve has been used. The standard curve has been built with different log concentrations of cDNA miR-seps 5, the equation of which follows the format y=mx + b. In this equation, the y and the x represent the axis of the graphic, the m represents the slope of the line and the b represents the intercept. This equation, or more specifically $log\,copies = \frac{Cq-b}{m}$, has worked as a constant and measurer for the samples. Since each experiment requires two µL of the extraction cDNA, to atone for the volume the value obtained was subsequently divided by two, thus obtaining the results in log copy number/µL.

Data analysis

In order to discern whether there is a significant difference between the manual and semi-automatic procedures, the HOT and TAT times were compared using a Student's t-test. The extractions must have normal distribution and homoscedasticity, which is the variance of the errors is constant over time. To test the normality a Shapiro-Wilk test was performed, and the Bartlett test was used to assess the homoscedasticity. In case the samples did not follow these conditions, the test would have been changed to a non-parametric Mann-Whitney U test.

As for the other values in the project, there have been two separate tests to assess the small RNA quantity (represented in ng/µL) and RNA quality (represented in A260/280 ratio). To compare the means of the two variables between the two methods, a Student's t-test (specifically a Welch's student's t-formula) was planned if the data were to be normal and homeostatic. However, the conditions were not met, and the test used was a non-parametric Kruskall-Wallis. The same line of thought has been applied to the absolute quantification (represented in log concentration) data.

All the statistical analyses have been performed using the program RStudio (version 1.3.959). The significance levels for all the tests performed have been of 0.05. For all the tests performed there were two hypotheses, the null hypothesis (H_0) indicates no significant difference between the two methods. On the other hand, the alternative hypothesis (H_1) indicates is that there is a significant difference. Other statistical values, namely the interquartile range and the median, have also been considered during the comparison.

Results

Time management

Judging by the limited time for diagnosis in the case of sepsis, the time for the methodology (TAT) is critical for the survival of the patient. It is also important to take HOT into account, given that the less time the investigators touch the extractions, the less contamination there will be. For that reason, the HOT and TAT have been analysed in the two extraction methods. Both times presented a normal distribution (Shapiro-Wilk test p-value > 0.05) and homoscedasticity (Bartlett test p-value > 0.05), therefore a Student-t test was used to see if there was a significant difference.

For the HOT, the test rejected the H_0 (p-value < 0.05), which means there was a significant difference between the manual and semi-automatic extraction methods regarding the HOT. The same result came out for TAT (p-value << 0.05). In the means of both times (Table 1), it is clearly visible the breach between the two extraction methods.

Table 1. Means of HOT and TAT for both extraction methods from all the extractions performed (n=54).

	HOT	**TAT**
Manual	37 min 20 sec	61 min 45 sec
Semi-automatic	52 min 30 sec	101 min 25 sec

Quantity and quality analysis

To assess the constitution of the extracted RNA obtained through both different extraction methods, a control of the small RNA quantity and RNA quality was realised (Appendix 2 and Appendix 3). However, with the purpose of detecting if there was a significant difference between the two methods, these results were analysed through a series of statistical tests.

In the first place, the small RNA quantity obtained was tested. Out of all the non-spiked extractions performed (n=30), five pertaining to the semi-automatic method (5/15) were marked out of range in the assay. All these extractions' small RNA quantity values have been replaced with a constant value of 0.12 ng/µL (Appendix 3) through the LOQ/√2 equation.

The small RNA quantity values did not follow a normal distribution, as proved after performing a Shapiro-Wilk test (p-value of 0.004 < 0.05). Therefore, a Kruskal-Wallis test was applied to compare the two methods' quantities. The statistical test reported a p-value higher than the level of significance, consequently H_0 was accepted. This means that the two extractions methodologies had no significant difference in regard to the quantity of small RNA.

Since the small RNA quantity distribution was skewed, it was more useful to refer to the median rather than the mean. The mean loses its ability to provide the best central location for the data because the skewed data is dragging it away from the typical value, whereas the median is not that strongly influenced. In the non-spiked extractions, the median for the small RNA quantity of the manual method (n=15) was 0.29 ng/µL (IQR=0.07) while for the semi-automatic (n=15) was 0.36 ng/µL (IQR=0.30)(Figure 2A).

The distribution of the small RNA quantity in the samples (Figure 2A) showed that the semi-automatic method had a higher variance than the manual method. The manual extraction method had less variability within the values compared to the semi-automated extraction method.

On the other hand, the RNA quality did not follow the normal distribution as well therefore another Kruskal Wallis test was applied. Once again the H_0 was accepted (p-value of 0.32 > 0.05), meaning no significant difference between the two groups. The distribution in this case (Figure 2B) showed less discordance between the two methods. The medians were 1.37 (IQR=0.32) for the manual method (n=15) and 1.32 (IQR=0.24) for the semi-automatic (n=15).

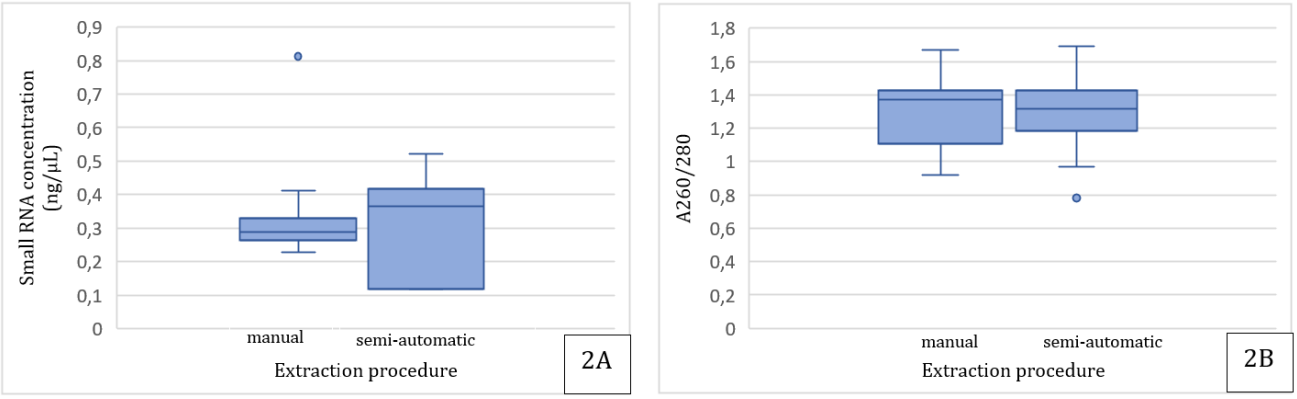

Figure 2 (A) Box plot representing the small RNA concentration in the non-spiked manual (n=15) and semi-automatic (n=15) extractions through the interquartile ranges. The error bars represent the confidence interval (95%) and the small blue circle is an outlier. The line in the middle is the depiction of the median. (B) Box plot representing A260/280 ratio in the non-spiked manual (n=15) and semi-automatic (n=15) extractions through the interquartile ranges. The error bars represent the confidence interval (95%) and the small blue circle is an outlier. The line in the middle is the depiction of the median.

Two-tailed RT-qPCR and absolute quantification

In order to absolutely quantify the non-spiked samples, a linear standard curve was built through serial ten-fold dilutions from 10^{11} to 10^{2} copies/µL of synthetic miR-seps 5. Out of all the original amounts of copies, only five (Appendix 4) had an acceptable amount of variability and were maintained. The resulting standard curve (Figure 3) has an efficiency of 111.1% due to a slope of -3.08. The coefficient of determination, R^2, has a value of 0.99.

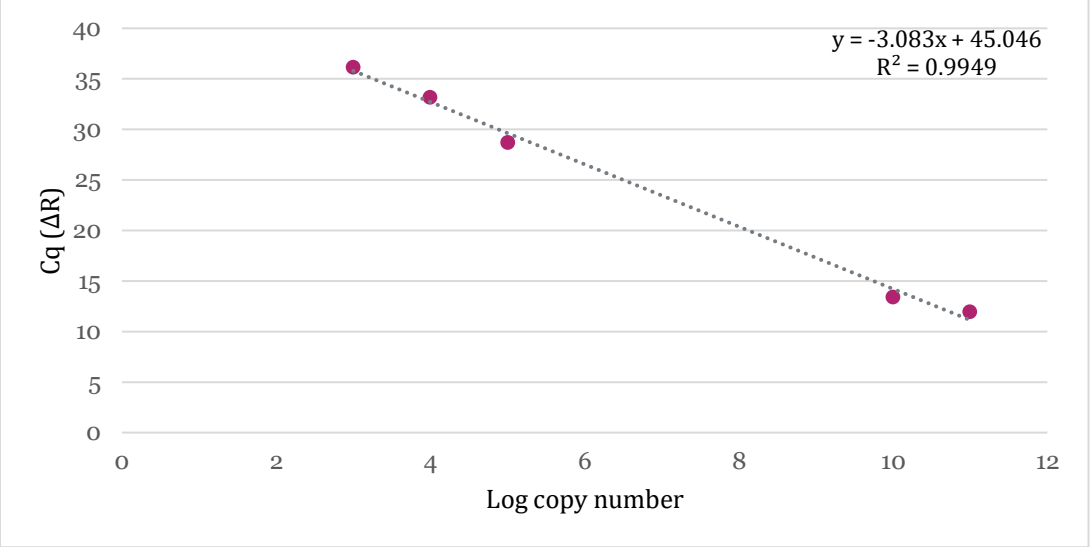

Figure 3. Standard curve built based on different log copy number of synthetic miR-seps 5. The equation of the curve and the R^2 value is shown in the right superior corner.

All of the non-spiked extractions (n=30) were subjected to absolute quantification through the equation obtained in the standard curve with the purpose of acquiring the log copy number. Out of all the non-spiked extractions (n=30) targeting the circulating endogenous miR-seps 5, seven of the manual (7/15) and three of the semi-automatic (3/15) were within the log copy number of the established standard curve (Table 2). The log copy numbers were obtained through the standard curve (Figure 3) equation, which would normally provide higher results than the ones displayed in Table 2. However, given the volume of cDNA used, the original values were divided by two in order to get the results in copies/µL.

Table 2. Two-tailed RT-qPCR data from the non-spiked extractions (n=15 manual, n=15 semi-automatic). The NS in the extraction name stands for non-spiked and the number beside it is the order in which the extractions were performed. The first 15 were made with the manual method, and the following 15 with the semi-automatic. The SD maximum value have been placed at 1, therefore no accepted extraction can surpass that value. All the results are the average of the accepted replicates (≤0.5 of difference), specified in the 2nd column.

Extraction	Accepted replicates (out of 3)	Average Cq value (ΔR)	SD (≤1)	Log copy number
NS1*	3	24.08	0.03	3.42
NS2	3	26.94	0.13	2.94
NS3	3	26.90	0.08	2.94
NS4	2	27.25	0.10	2.89
NS5	3	27.14	0.13	2.91
NS6*	3	26.38	0.08	3.03
NS7*	3	25.47	0.30	3.18

NS8*	3	23.42	0.16	3.51
NS9	3	27.64	0.05	2.82
NS10*	3	25.89	0.07	3.12
NS11	2	27.04	0.04	2.92
NS12	3	27.04	0.70	2.92
NS13*	2	24.52	0.15	3.33
NS14*	3	26.37	0.05	3.03
NS15	3	27.01	0.17	2.93
NS16*	2	26.42	0.20	3.02
NS17	3	27.13	0.11	2.91
NS18	3	28.44	0.05	2.70
NS19	3	28.75	0.06	2.65
NS20*	2	24.61	0.07	3.32
NS21	2	27.70	0.04	2.81
NS22	3	28.50	0.11	2.69
NS23	3	29.09	0.04	2.59
NS24	2	29.03	0.16	2.60
NS25	3	26.77	0.10	2.97
NS26	3	28.23	0.05	2.73
NS27	3	28.92	0.09	2.62
NS28*	2	23.83	0.06	3.44
NS29	2	28.63	0.14	2.66
NS30	3	28.80	0.07	2.64

*The highlighted results represent the quantifiable extractions

Moreover, to determine if there was a difference between the log copy numbers from the two extractions methods (Figure 4) a Kruskal-Wallis test was performed after the Shapiro-Wilk test rejected a normal distribution (p-value < 0.05). The Kruskal-Wallis test reported no significant difference between the two groups (p-value > 0.05). Other statistical measures, median and IQR, were also studied. For the manual extractions, the median of the log copy number was 2.94 (IQR=0.22). On the other hand, the semi-automatic extractions had a median of 2.70 (IQR=0.30).

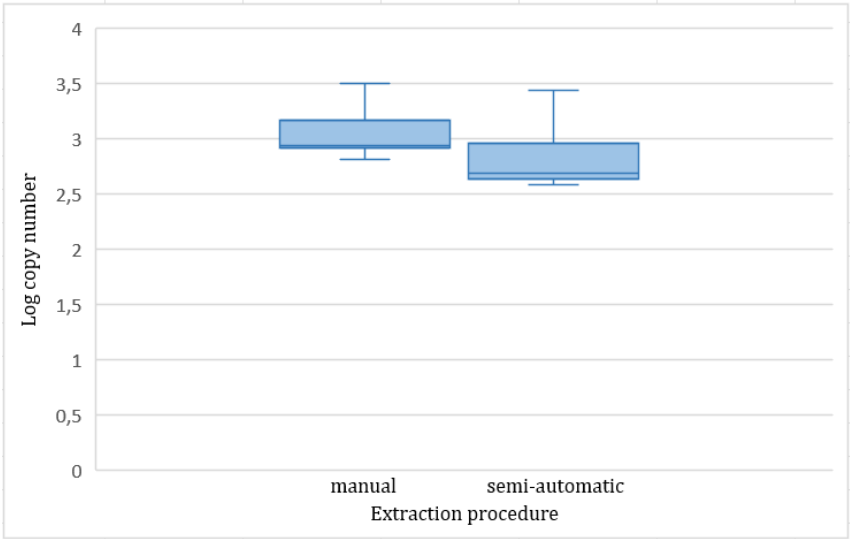

Figure 4. Box plot representing the non-spiked manual (n=15) and semi-automatic (n=15) extractions log copy numbers through the interquartile ranges. The error bars represent the confidence interval (95%). The line in the middle is the depiction of the median.

The melting curves of the endogenous circulating miR-sepis 5 (Figures 5A and 5B) showed a well-defined peak at the temperature of approximately 80ºC. More marked in the semi-

automatic one (Figure 5B) while slightly displaced in the manual (Figure 5A). There are no other observable secondary peaks. Similar results were observed in the melting curve from the standard curve, with one major peak at 80ºC (Appendix 4).

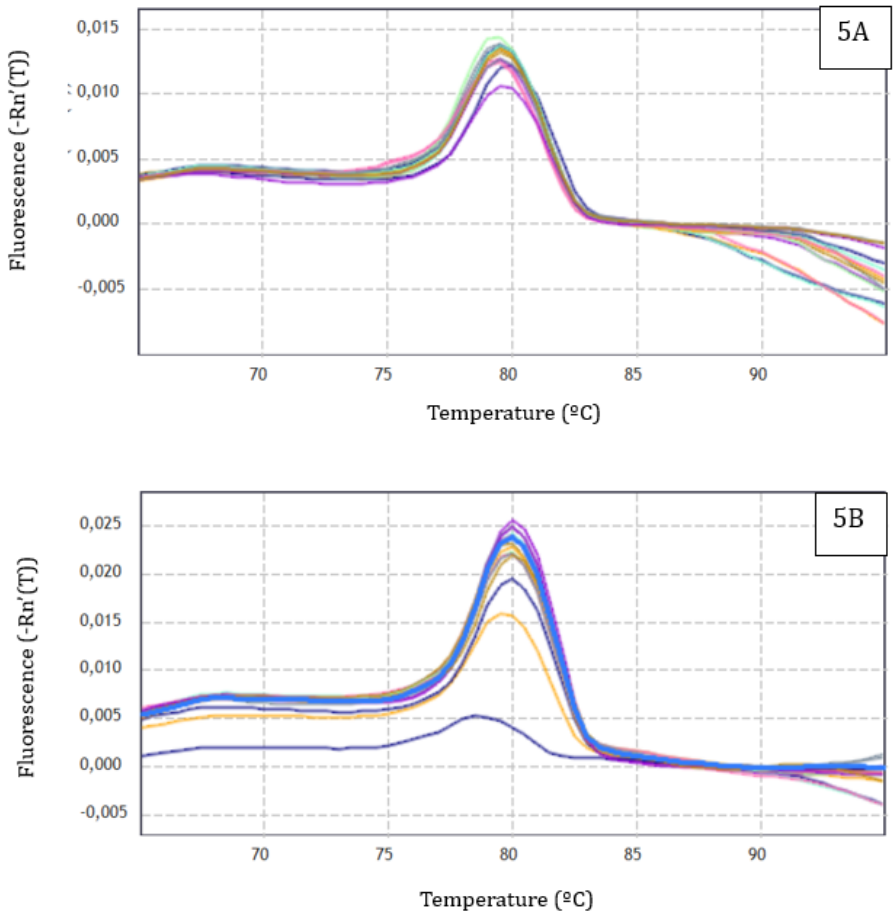

Figure 5 (A) Melting curve of the endogenous circulating miR-seps 5 cDNA from the non-spiked manual extractions (n=15). (B) Melting curve of the endogenous circulating miR-seps 5 cDNA from the non-spiked semi-automatic extractions (n=15).

Discussion

Nowadays, sepsis remains one major healthcare problem around the world. The main issue with this syndrome is that it develops quickly with little to no time to diagnose it correctly before it becomes serious. The lack of time and the variability of its symptoms made treating the syndrome extremely difficult (Oeschger et al., 2019). The current diagnostics methods usually involve blood cultures followed by species identification (Enroth et al., 2019) in order to recognize the microorganism responsible for the origin of the infection, however, this procedure takes too much time. New techniques are being studied with the purpose of developing an early diagnosis procedure in which the diagnostics time decreases, consequently improving the patients' prognosis. Biomarkers as a diagnostics tool is an emerging research field, especially when using miRNA as the biomarker (Esposito et al., 2017).

Due to the variability of sepsis, a single biomarker would not work to efficiently diagnose it. Therefore, a multiple biomarker kit was considered, eventually becoming the ultimate goal of

the research in this field. Nonetheless, there are many challenges to face before this goal can be achieved. Currently, the main issue is with the standardisation of the methods used for the investigation. Without this, comparing results and in due course making conclusions is increasingly difficult. Not to mention the already existing struggle to work with a biofluid like plasma and the low amount of endogenous miRNA, which has been emphasized in earlier studies (Backes et al., 2016).

In the present project, miRNA extracted from healthy plasma has been examined. The overall objective was to generate a standardised methodology for the extractions and quantification of the miRNA candidate, miR-seps 5. This candidate, among others, has been singled out in recent research about sepsis biomarkers not yet published. Two different RNA extraction methods have been used along with a novel technique for the detection and quantification of miRNA, the two-tailed RT-qPCR (Androvic et al., 2017).

Time management, quantity and quality analysis

The time management of the diagnostic process, as previously mentioned, remains an important factor of the process. The time comparison between the manual and semi-automatic extraction methods reported a significant difference (p-value << 0.05), with the manual method having the lower values (Table 1). However, it is worth mentioning that both methods' times (Table 1) depend on how many extractions were being performed simultaneously. In the particular case of the semi-automatic methodology, the machine may take as little as 20 minutes (2 samples) or as long as 40 minutes (10 samples). On the contrary, in the manual procedure, the fixed time is 24 minutes and 15 seconds, and the variant is the manual handwork. Also, it is necessary to consider the handling bias and the amount of time the samples were in contact with the investigator. The manual method may have lower values but the amount of interaction with the extraction could potentially increase the risk of contamination which is detrimental for the PCR methodology (Sharma et al., 2022).

Moving on to the small RNA quantity, in a weighty number (5/30) of the non-spiked extractions, the small RNA quantity came out as out of range for being too low (Appendix 3). Some of the extractions from the optimization also reported an out of range (Appendix 2), while none of the positive controls (Appendix 3) presented such low values. According to the protocol (Thermo Fisher Scientific, 2022), the minimum amount detectable is 0.5 ng. With this value, and with three µL of extracted eluted used for the Qubit microRNA assay, these undetectable quantities were replaced with a constant value of 0.12 ng/µL through the LOQ/$\sqrt{2}$ equation (Bergstrand & Karlsson, 2009). However, since the optimization extractions were not used in the statistical analysis, they were not replaced with a constant value.

In the field of molecular biology research, data lost because of the limit of quantification is a regular hindrance. Particularly, when new biomarkers are being assessed, the limit of detection (LOD) and limits of quantification (LOQ) are two customarily used measures that more often than not obstruct the estimation of the data collected. Seldom do researchers publish results that reach below LOD and/or LOQ, which produces a situation of incomplete data. Such lacking information can originate bias, inefficiency and cause the analysis to be more complex than it should be (Harel et al., 2014).

The LOQ/$\sqrt{2}$ equation is one of the options for the results to be included. As mentioned before there are divergent opinions regarding this kind of data, however, they were included in this study because it was reported that as the value of the biomarker escalates, so does the standard deviation (SD) of the predictive distribution. This points out to the conclusion that the alternation of the values higher than the LOQ is actually higher than the distortion of the value below the LOQ (Guo et al., 2010). Not to mention that the two-tailed RT-qPCR (Androvic et al., 2017) can detect concentrations below the threshold set for the Qubit® MicroRNA Assay

protocol (Thermo Fisher Scientific, 2022), therefore samples below the detection limit (Appendix 3) were processed further and candidate microRNA was detected in all samples.

The statistical analysis performed in the different small RNA quantities between the manual and semi-automatic extraction methods reported that there was no significant difference between them (p-value > 0.05). One option to improve the sensitivity (Groenewald, 2022) would be to increase the volume of extraction added to the assay. If, for example, the volume was increased to five µL, the LOD would be 0.1 ng/µL which is lower than the constants obtained using the LOQ/$\sqrt{2}$ equation. Therefore, it is worth considering the volume of extraction added to the assay when optimising the procedure.

The median of the quantity of small RNA did not differ significantly between the two extraction methods (Figure 2A). Very little has been experimented with the candidate miR-seps 5, however, compared with the few results known (Kasaras, 2022) some differences can be observed. For the manual extraction, the median in this study was 0.29±0.07 ng/µL. While previous results shown in Kasaras (2022) were at 0.12±0.16 ng/µL. In the semi-automatic extraction method, the present median was 0.36±0.30 ng/µL while the previous (Kasaras, 2022) value stood at 0.29±0.29 ng/µL.

In the RNA quality values (Figure 2B), the present study had medians of 1.37±0.32 for the manual and 1.32±0.24 for the semi-automatic. In the previous study (Kasaras, 2022), the means were 1.91±1.84 for the manual and 0.91±0.19 for the semi-automatic.

It is worth mentioning that in this previous study (Kasaras, 2022), the small RNA quantity as well as the RNA quality had normal distribution, unlike the present study, consequently the values described were the mean rather than the median so that might explain the difference between the two studies. Another possible reason why the values were different is the natural biological variability that comes with using plasma from different persons (Taylor et al., 2010). Another source of variation could be the number of extractions performed, the past study had fewer non-spiked extractions performed (n=5 manual and n=5 semi-automatic). Not to mention the variation displayed, comparing the SD from Kasaras (2022) with the IQR from the present one, there is a clear breach between the values. The present study has a generally lower variation (Figures 2A and 2B), which proves beneficial for the reproducibility of the experiment. There is also the fact that the whole fraction of RNA, including miRNA, was extracted. Therefore, in that regard, the results are comparable to studies that have investigated RNA extraction in general and not only the miRNA candidate. For example, Lindeberg (2022) study in contrast reported significantly higher median quantities in both extraction methods.

The first part of the experiment, the extractions, are usually the main source of variation in multiple molecular biology assays and other experiments (González et al., 2021). As much as miRNAs have been chosen as a biomarker for their stability (Benz et al., 2016), RNA molecules are still highly susceptible to degradation once the tissue has been extracted from the organism. Therefore, certain measures need to be performed to assure a minimum degradation (Eikmans et al., 2013).

Simple precautions and application during the experiments in the laboratory can decrease or avoid altogether the risk of RNA degradation, such as good aseptic techniques, making sure that all the material used is free of RNAses and periodical changes of the nitrile gloves. Additionally, all the water used for the preparation of the mixtures or other reagents should be RNA-se-free (Williams et al., 2010). All these precautions have been performed in the current study.

Furthermore, the choice of extraction method is also important (Fitzpatrick et al., 2021). This study has used a column-based kit as the extraction method, however, there is an ongoing discussion about the merits and disadvantages of the column-based kits compared to the more

traditional phenol-chloroform-based (Toni et al., 2018). Both have advantages and disadvantages, kits tend to be faster but can be less economical than the traditional method, especially in a large number of samples (Toni et al., 2018). However, the column-based ones usually provide higher-quality elutions and do not have a very high extraction variation (Ban et al., 2022), as shown in the current study. On the other hand, the traditional method is reported to work better in small volumes of extractions by providing 2.4-93 times more RNA than the kits (Deng et al., 2005) and has the advantage of favourable extraction recovery and costs (Ban et al., 2022). In this study, it was considered that the advantages outweigh the disadvantages of the column-based kits, mainly because of the time employed for the extractions as well as the need for the miRNA to be as pure as possible for downstream analysis, rather than favouring the quantity of it. And out of all the disponible kits to perform with, and as mentioned in the introduction, multiple studies (Sriram et al., 2021; Androvic et al., 2019; Garcia-Elias et al., 2017) have deemed the miRneasy Serum/Plasma kit (QIAGEN) as the best option.

The fraction composed of miRNA after RNA extraction is different depending on the biofluid used. If plasma is the biofluid used, miRNA can be found with a median of 80.9% of the overall composition. On the other hand, if serum is used, the miRNA median of the overall composition is reduced to 54.4% (Max et al., 2018). The % in the current study was not measured, however, the information is useful for reference in the amount of miRNA in each biofluid and their consequences in the results. Moreover, in order to obtain plasma, centrifugation with added anticoagulants, citrate in this case, can be used. On the contrary, serum is obtained through the centrifugation of coagulated blood. Technically speaking, using serum is easier however it is reported that as a consequence of coagulation, platelets let out numerous extracellular vesicles containing miRNA, which alters the conformation of RNA in extracellular blood. Because of this, plasma is usually preferable (Galvanin et al., 2019).

Even then, plasma is still considered a challenging fluid. Depending on the biofluid, the composition changes and it is an important factor to take into account (Bryzgunova et al., 2021). In the case of blood human plasma, proteins are found in high concentrations, specifically of 7.2 g/dL (Blanco & Blanco, 2017). According to these levels, the chaotropic agents should have enough volume to separate the miRNA from the proteins. Also, depending on the starting volume the amount of denaturing solutions needed for complete dissociation of miRNA from the protein complexes increases or decreases. Joined with the starting volume, these are the most variable steps throughout the different protocols (Moldovan et al., 2014). The reagents in this project used to separate the plasma components from the RNA have been calculated according to the starting material, 100 μL, and to previous students' thesis (Groenewald, 2022; Kasaras, 2022; Nilsson, 2021) to build an optimised methodology.

RNAses are abundant in human plasma, which makes it difficult for the circulating RNA to get protection and avoid degradation. Protection against this usually occurs in two ways, one of them is particularly visible in miRNA, they are found to be in a soluble state and included in ribonucleoprotein complexes. It is also possible that they are included in other particles such as low-density lipoproteins (Vickers et al., 2011). On the other hand, RNA can be found in extracellular vesicles (Momen-Heravi et al., 2018). The problem with this is that they are so intrinsically joined that separating them during the extraction is distinctly difficult, leading to a decrease in the purity of the extractions. This is one possible explanation as to why none of the extractions performed during the whole project (Appendix 2 and 3) has reached the generally accepted A260/280 value of 2 for pure RNA (Desjardins & Conklin, 2010).

In this project, despite aiming for earlier diagnostics of sepsis, plasma from healthy blood donors has been used since it is necessary to start with the biological fluid that is least challenging to work with. Plasma itself is already difficult (Galvanin et al., 2019), but since the two-tailed RT-qPCR is a new technology (Androvic et al., 2017) that, until recently, had never been tried in any biological fluid, plasma from healthy individuals is better for the first experiments. Plasma from

septic patients has white blood cells, platelets, and another viscosity (Dewitte et al., 2017) making it more challenging to extract miRNA. Once the process of miRNA detection in plasma with two-tailed RT-qPCR works on plasma from healthy individuals, the research can proceed to work with plasma from septic patients.

One issue to take into account with the plasma used in this study is that it has been in storage for a prolonged period of time, over seven years. In terms of storage, certain actions may affect the miRNA concentration (Silva et al., 2015). For instance, numerous freeze-thaw cycles may cause that. Also, long-term storage. According to a review (Bryzgunova et al., 2021), when it is not much time (>20 months) no significant differences were observed in storage between -80ºC and -20ºC, however, during the mark of six years a clear reduction in miRNA levels was observed, one that only grew as time passed by. This may explain why the small RNA quantities obtained were much lower than normal (Appendix 2 and 3), since in human plasma blood the usual amount of small RNA is between 1.91 and 5.29 ng/µL (Chomczynski et al., 2016).

Also, regarding the small RNA quantity, another factor to consider is the centrifugation force. Despite not having a significant difference between the two extraction methods in small RNA quantity, the semi-automatic extractions had generally higher values (Figure 2A). There seems to be a positive correlation between small RNA and relative centrifugal force (RCF). In a previous study (Li et al., 2020), when groups were compared using different g, the one with the highest small RNA yield was the one which had the highest g. This is not surprising, taking into account that centrifugation is performed in order to dissolve the possible aggregates found in the samples. The maximum RCF used during the manual extraction is 14100, while in the QIAcube the maximum RCF is 12000 (QIAGEN, 2018). However, and against the results of Lin et al. (2020), in this study both the spiked and non-spiked semi-automatic extractions possessed higher small RNA quantities than the manual extractions (Appendix 3). On the other hand, the optimization extractions (Appendix 2) did have higher small RNA quantities in the manual extractions than in the semi-automatic ones.

Two-tailed RT-qPCR and absolute quantification

With the purpose of creating a standardised methodology for the extraction and quantification of the miRNA candidates, which is a major interest within the sepsis biomarkers research, methods previously used in thesis projects were implemented. Consequently, previous students' (Groenewald, 2022; Kasaras, 2022; Nilsson, 2021) values regarding reagent volumes and concentrations were used. In order to quantify the miR-seps 5 from the non-spiked extractions (n=30), a standard curve (Figure 3) was built through serial ten-fold dilutions using synthetic miR-seps 5 (Integrated DNA Technologies). A standard curve is a crucial step in every qPCR experiment when using the absolute quantification method. It has the function to determine the efficiency, sensitivity, reproducibility and working range of the assay. It is recommended for the standard curve to have at least three replicates per concentration and a minimum of five log concentrations for the detection to be as accurate as possible (Green & Sambrook, 2018).

Originally, with the ten different log concentrations included, the efficiency of the standard curve surpassed the acceptable values (Taylor et al., 2010) by 10%, 121. 1%, with a R^2 value of 0.96. The Cq values from some points of the original standard curve, namely log concentrations 2, 6, 7, 8 and 9, did not provide a good linear correlation between the values and were consequently removed from the final product (Figure 3). The efficiency of the ending product in the current study, after removing the more variant points, was 111.1%, which is considered slightly higher than the acceptable superior limit of 110% (Taylor et al., 2010). The R^2 value of 0.99 states a good linear correlation among the values inserted in the standard curve. Previous studies working with miR-seps 5 (Kasaras, 2022) had similar problems regarding the qPCR efficiency, with a final value of 116%.

Efficiency is an important factor of every PCR reaction and is described as the amount of molecules targeted that are copied in one cycle (Lalam, 2006). The final PCR efficiency builds from different factors. First off, there is the assay execution, including primers and template sequences among others. Second, there is the sample matrix which is possible to have inhibitors within or other molecules from the sample. Third, the reagents used during the assay and their concentrations. Fourth and final, competing reactions (Svec et al., 2015).

The expected efficiency is of 100%, slope of -3.33, in a series of ten-fold dilutions. This originates from the supposition that during each cycle, the molecules double. However, in reality, this kind of perfect doubling is highly unusual. In most cases where the efficiency surpasses 100% the reason behind is inhibition (Nolan et al., 2006). It tends to affect the more concentrated templates, provoking a disruption in linearity. If these samples are maintained in the standard curve it leads to a reduced slope and too-high efficiency (Svec et al., 2015).

The inhibition is usually caused by the remaining phenol from the extraction steps. This remaining phenol has the ability to inhibit PCR reactions through the denaturalization of polymerases (Unger et al., 2019). These kinds of consequences have been observed in extractions where the phenol levels were as low as 0.02% (Loffert et al., 1997). Detection of phenol and other contaminants can be perceived with a spectrophotometer, measuring either the A230/260 or the A260/280. In the current study there was no phenol used during the extraction but there was ethanol, which can also inhibit the PCR reaction (Schrader et al., 2012). Another reason why the RNA quality values (Appendix 2 and Appendix 3) may have come out lower than the acceptable values. Although filtered tips for the pipettes were used in every experiment performed during the project with the purpose of avoiding this situation. Especially taking into account how the PCR methodology is really sensitive and other contaminant molecules may affect the results.

It is also confirmed that the efficiency can be aided by the number of replicates performed for every log concentration. With two replicates, accuracy improved by 41%, moreover using three replicates it improved by 68% (Svec et al., 2015). However, given that three replicates have been used in the current study and none have been removed, this should not have caused any major problems with the efficiency.

Despite this, the absolute quantification of the non-spiked extractions (n=30) all came out within relatively good levels considering that they are circulating miRNA in plasma. Normally since miRNA is such a small molecule it can take multiple cycles to be able to detect it, especially in non-spiked samples. Nevertheless, all the Cq of the non-spiked extractions were below 30 (Table 2), as were the ones in the spiked candidate extractions (Appendix 3). On the other hand, the optimization extractions performed with miR-210 were higher and above 30 in some extractions. The differences in the Cq values between the optimization and the candidate extractions may be ascribed to the difference in the miRNA used. Cq values start to be considered unreliable when they reach 30, in the particular case of miRNA it is accepted till 35 (Izquierdo et al., 2014) but these kinds of results do not carry much weight. Though it is worth taking into account that the miRNA introduced in the qPCR has been transformed into cDNA during the RT reaction.

All the extractions performed (n=54), as well as the standard curve, were performed in three replicates in the qPCR machine, plus three NTC replicas per assay to ensure no contamination during the two-tailed RT-qPCR process. However, some of the extraction replicates had to be removed because of technical variance, in the case that there was no correlation between the triplicates the whole value was removed and the extraction repeated. There are different opinions regarding the removal of replicates, normally it is suggested that when the Cq value differentiates by more than 0.5 (Dijkstra et al., 2012) it should be removed to ensure valid data.

This is the principle applied in the project, although other opinions (De Ronde et al., 2017) maintain that applying this to the whole Cq data may cause the loss of valid data points.

All of the 30 non-spiked extractions were subjected to quantification through the two-tailed RT-qPCR, 10 extractions reported quantifiable results (Table 2) within the final standard curve values. Of the 10 mentioned, seven belonged to the manual extractions' procedures (7/15) and three to the semi-automatic (3/15). The statistical analysis reported no significant difference (p-value > 0.05) between the two extraction methods, however, given that these are the first stages of investigation in a novel research field further investigation is suggested. The amplification of the cDNA was clearly visible (Table 2) and therefore the miR-seps 5 can be amplified using the two-tailed RT qPCR assay. Both methods had relatively low variation in the absolute quantification (Figure 4), though the manual displays more consistency with an IQR of 0.22 in front of the semi-automatic with an IQR of 0.30. Which may prove to be troublesome for the reproducibility of the experiment. The Cq values (Appendix 3) were acceptable for miRNA values, however, they could be reduced in order to gain accuracy. One way for this would be to increase the qPCR reaction volume with approximately 10 µL extra since a higher cDNA template volume could decrease the number of cycles needed for detection.

Moreover, it is worth mentioning the risk of false positives in the qPCR analysis. Factors like hemolysis or DNA contamination may increase this risk. To minimise it, the two-tailed RT-qPCR possesses an RT primer with a stem-loop (Androvic et al., 2017) which results in a preference for RNA/DNA duplex formation and thus avoids annealing to other molecules. One study (McDonald et al., 2011) reported that hemolysis can simulate higher miRNA concentrations than the ones in reality. Not all miRNA species react the same way, but out of the miRNA studied a significant number was shown to increase from their natural concentration. Although it is not currently possible to know if this would affect miR-seps 5 the same way as there is no knowledge of this miRNA found in erythrocytes. A positive insight is that the miRneasy Serum/Plasma Advanced kit (QIAGEN) was not reported to hemolysis in the extractions (Nordén, 2020).

In previous studies with miR-seps 5 (Kasaras, 2022), the absolute quantification values differed significantly from the current study. While Kasaras (2022) had the extractions log copy number between seven and eight, the current study log copy numbers were between 2.59 and 3.51 (Table 2). These changes may be caused by the differences in efficiency and R^2 value of the standard curves.

Regarding the melt curve of the extractions (Figures 5A and 5B), both had a defined peak around 80ºC of temperature. Similar results were observed in the melt curve from the standard curve (Appendix 4). This showed a good specificity of the different primers used throughout the two-tailed RT-qPCR (Taylor et al., 2010). No other secondary peaks were observed. The melt curve is used to determine if the intercalating dye qPCR assays have produced a single, specific product (Bruzzone et al., 2013). It ensures that the primers have not joined with one another, a phenomen named primer dimers. Consequently, a single peak can be interpreted as a single amplicon found in the melting analysis.

Ethical considerations

The present study remains focused on the most novel techniques that researchers within the area of molecular diagnostics are studying nowadays. It pertains to the frontier of the research in this field, which comes with positive prospects for the future but also with its own hindrances, mainly the fact that given its newness there is almost no published articles to compare with and eventually draw solid and valid conclusions. Nevertheless, and as much as the information displayed until now is nothing astounding, is the beginning in the right direction.

As the investigation of sepsis diagnostics advances, the ultimate goal of an early and efficient diagnostic method is nearer and more achievable. It is estimated that every year more than 30 million people are diagnosed with sepsis, with 5 million of the total succumbing to death (Caraballo & Jaimes, 2019). While the survivors deal with long-term repercussions that remain mostly unknown and are being studied (Scherag et al., 2017), however, they are known to cause additional morbidities and rehospitalization often during the first years after suffering from the syndrome (Shankar-Hari & Rubenfeld, 2016).

Having an accurate and swift protocol for the diagnosis would produce an unimaginable effect on the survival numbers of the syndrome, not to mention on the long-lasting effects found in the survivors. As an added benefit, it would provide aid in the field of antibiotics. The major treatment for sepsis is based on antibiotics, nowadays physicians normally apply a broad spectrum of antibiotics (Martínez et al., 2020). If the physicians were to know exactly which microorganism originated the syndrome, it would greatly reduce the amount of antibiotic used as well as the bacterial resistance that may ensure from the application of multiple antibiotics.

Conclusions

This study investigated two main questions, that is to say, if the semi-automatic methodology could provide higher quantity and quality miRNA than the one provided by manual means, and if the two-tailed RT-qPCR was capable of detecting the candidate miR-seps 5. No significant difference was observed in the small RNA quantity and the RNA quality between the two extraction methods. On the opposite side, the time management results did report a significant difference, although it is worth considering that the number of extractions being performed at the same time may affect the final time. However, overall the manual method displayed less variance which might be beneficial for future reproducibility.

The two-tailed RT-qPCR has proved to be able to accurately detect and quantify the miR-seps 5 coming from the non-spiked extractions of 100 µL of plasma. The creation of the linear standard curve produced some issues with efficiency, which may suggest a more careful approach during the procedure to avoid contamination as well as make sure that the primers are adequate for the target. Though the melt curve showed a single amplicon which indicates a good specificity of the primers. A troublesome element to take into account is the extremely low number of log concentrations that miR-seps 5 displays in the results. Possible ways to increase the amount of template used during the two-tailed RT-qPCR should be further investigated in order to make sure that the low quantity does not cause problems with assay efficiency.

A successful miR-seps 5 extraction using manual and semi-automatic methods, followed by quantity and quality control and the detection through the two-tailed RT-qPCR has been performed in this project. Ultimately, and with further investigations, miR-seps 5 may be extracted, amplified and detected from the plasma provided by the biobank. Which, should it be successful, would mean the inclusion of the miR-seps 5 to the SepsIT® multimarker sepsis diagnosis panel.

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Appendix 1

For the reverse transcription the equipment used is as follows: GrandScript cDNA FreePrim Kit (TATAA Biocenter), two-tailed RT primer for the miR-210 and miR-seps 5 (Integrated DNA Technologies), filtered pipette tips, 0.2 mL PCR tubes and nuclease-free water. The sequence of the RT primer for miR-210 is 5′–AAATACACGTGCTAGACTCTACACACTTACTATAAGTAAGTAACCTATTAGAACTCAGC–3′, the one for miR-seps 5 will not be disclosed as it is confidential information.

Previous preparations include thawing all frozen components – including all kit components along with the RT primers, standard curve, synthetic spike-in miRNA and miRNA samples. Mix each component thoroughly and centrifuge it to collect the content. Place all components, including the GrandScript RT Enzyme, on ice.

1. Add the following components (Table 1), for one reaction, to a 0.2 mL PCR tube while placed on ice. For each extraction there should be a different single reaction.

Table 1. Components and volumes of the RT reaction.

Component	**Single reaction**
GrandScript FreePrime Mix, 5x	2 µL
GSP Enhancer, 10x	1 µL
RT Enzyme, 2 µM	0.5 µL
Two tailed RT Primer, 2 µM	2.5 µL
miRNA sample	4 µL
Final volume	10 µL

2. When preparing standard curve samples: add 1 µL of synthetic miRNA along with 3 µL of RNAse-free water instead of 4 µL of miRNA sample.

3. Vortex gently and centrifuge to collect content.

4. Incubate through the PCR machine (Table 2). In this particular case, a Biometra Thermocycler TProfessional Basic 96 (Montreal Biotech) was used.

Table 2. Temperature and time of each stage that define the incubation cycles for the RT reaction.

Temperature (ºC)	**Time (min)**
42	45
85	5

4	Hold

For the qPCR reaction the equipment is as follows: SYBR GrandMaster Mix (TATAA Biocenter), 1.5 mL microcentrifuge tube, 0.2 mL PCR tubes, forward and reverse primers for the miR-210 and miR-seps 5 (Integrated DNA Technologies) filtered pipettes tips, 96-well PCR plates, nuclease-free water and the suitable cDNA template for each sample. The forward primer for miR-210 has the sequence 5′-GGCTACTCCCTCGC-3′ and the reverse has the sequence 5′-AGCCCCTGCCCAC-3′, the ones for miR-seps 5 will not be disclosed as it is confidential information.

Previous preparations include thawing all frozen components – including all kit components along with forward and reverse primers and the cDNA samples (if these were frozen after the RT procedure). Mix each component thoroughly and centrifuge to collect the content.

1. Make a master mix for x number of reactions (+10%).

2. Each sample runs in triplicates on the PCR plate. Based on this, the mix for each sample will contain enough for 3.5 reactions.

3. Master mix – add the following components (Table 3) to a 1.5 microcentrifuge tube.

Table 3. Components and volumes for the Master Mix of the qPCR reaction. Each time the reaction is performed, the number of samples is different, and therefore each time there will be different number of reactions to be calculated.

Component	**Single reaction**	**3.5 reactions**
SYBR GrandMaster Mix, 2x	5 µL	17.5 µL
Forward primer, 10 µM	0.2 µL	0.7 µL
Reverse primer, 10 µM	0.2 µL	0.7 µL
Nuclease-free water	2.4 µL	8.4 µL
ROX dye, 500 µM	0.2 µL	0.7 µL
Final volume	8 µL	28 µL

4. Reaction mix – add the following components (Table 4) to a 0.2 mL PCR tube (one tube per sample, 3.5 reactions per tube).

Table 4. Components and volumes for the mixture that is to be placed in the PCR-plate wells. For the samples, each well will have 8 µL and for the standard curve 9 µL.

Component	**Single reaction**	**3.5 reactions**
Master mix	8 µL	28 µL
cDNA	2 µL	7 µL

Final volume	10 µL	35 µL

5. Dispense each reaction mix into three wells of the 96-well PCR plate. Cover the plate with a transparent plastic cover, making sure that all the wells are shielded.

6. Gently vortex and centrifuge to collect content.

7. Put the PCR plate into the qPCR machine with the selected cycling protocol (Table 5). In this case, a AriaMx Real-time PCR System (Agilent Technologies) was used.

Table 5. Temperatures and duration of the cycling protocol for the qPCR reaction.

Stage 1	**Cycles: 1**	
95 ºC	30 seconds	
Stage 2	**Cycles: 40**	
95 ºC	5 seconds	
60 ºC	15 seconds	
72 ºC	30 seconds	Data collection
Stage 3	**Cycles: 1**	
95 ºC	15 seconds	
60 ºC	30 seconds	
95 ºC	15 seconds	

Appendix 2

Table 6. Raw data of the spiked miR-210 extractions (n=12) regarding the small RNA quantity, RNA quality and miRNA detection. All the results are the average of the replicates (n=3) performed. The "M" stands for the extractions performed by the manual method and the "A" for the semi-automatic. The first six extractions pertain to part 1 of the optimization, while the following six are from part 2. The Cq values are represented as the mean of the three replicates performed. The SD maximum value have been placed at 1, therefore no accepted extraction can surpass that value.

Extraction method	**Spike-in copy number**	**Small RNA quantity (ng/µL)**	**RNA quality (A260/280)**	**Cq value (ΔR)**	**SD (≤1)**
M	5	0.60	1.47	30.31	0.18
M	6	0.52	1.98	33.26	0.39
M	7	OOR*	0.85	22.47	0.19
A	5	OOR*	1.37	26.56	0.11
A	6	OOR*	1.03	31.26	0.13
A	7	OOR*	1.46	27.34	0.15
M	5	0.98	1.22	30.60	0.19
M	5	0.87	1.24	29.99	0.50
M	5	0.88	1.15	31.49	0.14
A	5	0.45	1.45	32.33	0.86
A	5	0.35	1.14	33.24	0.71
A	5	OOR*	1.39	31.56	0.54

*Out of range (too low)

Appendix 3

Table 7. Raw data of the spiked miR-seps 5 extractions (n=12) regarding the small RNA quantity, RNA quality and miRNA detection. All the results are the average of the replicates (n=3) performed. The "M" stands for the extractions performed by the manual method and the "A" for the semi-automatic. The Cq values are represented as the mean of the three replicates performed. The SD maximum value have been placed at 1, therefore no accepted extraction can surpass that value.

Extraction method	Spike-in copy number	Small RNA quantity (ng/µL)	RNA quality (A260/280)	Cq value (ΔR)	SD (≤1)
M	5	0.28	1.43	15.27	0.18
M	6	0.21	1.46	21.41	0.13
M	7	0.22	1.76	20.72	0.04
A	5	0.43	0.33	27.87	0.45
A	6	0.44	0.18	24.55	0.19
A	7	0.46	1.11	18.06	0.36
M	5	0.23	1.40	20.21	0.17
M	5	0.24	1.44	15.90	0.06
M	5	0.26	1.42	18.76	0.57
A	5	0.33	1.17	21.47	0.18
A	5	0.40	1.36	29.92	0.04
A	5	0.40	0.75	26.17	0.45

Table 8. Raw data of the non-spiked extractions (n=30) regarding the small RNA quantity and RNA quality. All the results are the average of the replicates (n=3) performed. The "NS" in the extraction name stands for non-spiked and the number beside is the order in which they were performed. The first 15 were made with the manual method, and the following 15 with the semi-automatic.

Extraction	Small RNA quantity (ng/µL)	RNA quality (A260/280)
NS1	0.81	1.20
NS2	0.31	1.03
NS3	0.41	1.26
NS4	0.23	1.50
NS5	0.27	1.67
NS6	0.26	1.06
NS7	0.34	1.44
NS8	0.28	1.47
NS9	0.40	0.92
NS10	0.29	1.39
NS11	0.31	1.41
NS12	0.25	1.16
NS13	0.29	1.39
NS14	0.25	1.37
NS15	0.28	1.01
NS16	0.12*	1.56
NS17	0.36	1.17
NS18	0.41	0.78
NS19	0.38	1.20
NS20	0.12*	1.69
NS21	0.12*	1.45
NS22	0.12*	1.31
NS23	0.12*	1.38
NS24	0.27	1.29

NS25	0.31	1.06
NS26	0.52	1.43
NS27	0.42	1.42
NS28	0.42	0.97
NS29	0.46	1.42
NS30	0.42	1.32

*Originally out of range for being too low. Constant value applied using the LOQ/$\sqrt{2}$ equation.

Appendix 4

Table 9. Standard curve original values.

Starting amount (copies)	Replicate	Well	Cq value (ΔR)
10^{11}*	1	A1	12.48
10^{11}*	1	A2	12.54
10^{11}*	1	A3	12.76
10^{10}*	2	A4	13.87
10^{10}*	2	A5	13.86
10^{10}*	2	A6	13.73
10^{9}	3	A7	13.78
10^{9}	3	A8	13.92
10^{9}	3	A9	13.49
10^{8}	4	A10	16.70
10^{8}	4	A11	16.73
10^{8}	4	A12	16.77
10^{7}	5	B1	30.39
10^{7}	5	B2	30.59
10^{7}	5	B3	30.60
10^{6}	6	B4	34.85
10^{6}	6	B5	30.60
10^{6}	6	B6	34.47
10^{5}*	7	B7	37.76
10^{5}*	7	B8	37.97
10^{5}*	7	B9	37.75
10^{4}*	8	B10	33.42
10^{4}*	8	B11	32.90
10^{4}*	8	B12	32.56
10^{3}*	9	C1	37.63
10^{3}*	9	C2	37.24
10^{3}*	9	C3	37.52
10^{2}	10	C4	No Cq
10^{2}	10	C5	36.78
10^{2}	10	C6	32.32

*The highlighting signals the replicates that were used to do the final standard curve.

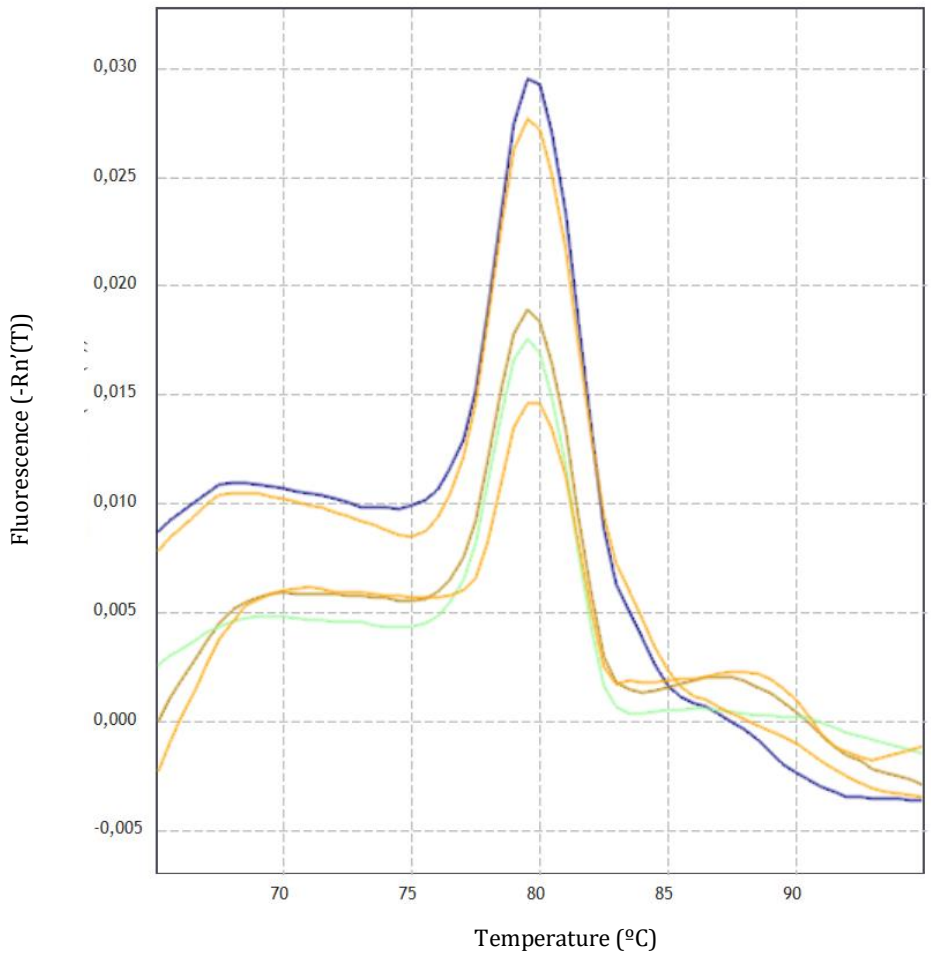

Figure 1. Melt curve of the final standard curve, after removing the values that were to different between replicates.