

Sepsis – Absolute Quantification of MiRSeps- 7 by using Two-Tailed RT- qPCR

Bachelor's Degree Project in Bioscience

Second Cycle 30 credits

Spring term 2024

Student: Jonas Paju, a19jonpa@student.his.se

Supervisor: Anna-Karing Pernestig, anna-karin.pernestig@his.se

Examiner: Helene Lindholm,
helene.lindholm@his.se

Abstract

Sepsis is a life-threatening condition, which in time damages the patient's organs. The quick sequence of events that could let a patient's condition go from stable to severe in a few hours requires physicians to establish an early and accurate diagnosis. The research on new potential biomarkers is in full throttle. miRNA has elevated its potential as a diagnostic biomarker and may be included in a multiple-biomarker panel for sepsis diagnosis. In this project, the aim was first to evaluate if miRSeps-7 could be detected and quantified in human plasma by using the Two-tailed RT-qPCR method. Secondly, to compare the efficiency of two total RNA extraction methods (manual and robotic (Qiacube) extraction). This comparison was based on measuring the Hands-On Time (HOT) and Turnaround Time (TAT) during the extraction processes, along with analyzing the Cq values obtained from the qPCR reactions. Additionally, a QC check was performed on all samples after RNA extraction. No significant differences were discovered between the two extraction methods in terms of time management (e.g. HOT & TAT) nor the quality assessment, with quantification cycles (Cq). The small sample size was a major disadvantage. Amplification was achieved on 7/20 samples with the Two-tailed RT-qPCR. However, it has yet to be confirmed that the amplified product was miRSeps-7.

List of abbreviations

cDNA - Complimentary deoxyribonucleic acid

CI - Confidence interval

Cq - Quantification cycle

CRP - C-reactive protein

DNA - Deoxyribonucleic acid

HOT - Hands On Time

IQR - Interquartile range

LOQ - Limit of Quantification

miRNA - Micro-ribonucleic acid

NGS - Next generation sequencing

PCT - Procalcitonin

RNA - Ribonucleic acid

-RT - No-reverse Transcription

RT - Reverse Transcription

RT-qPCR - Reverse transcription quantitative Polymerase Chain Reaction

TAT - Turnaround time

POCT - Point-of-care-testing

Table of contents

Introduction	1
MicroRNA as biomarker	1
Measurement of miRNA expression.....	2
qPCR, Quantification cycle (Cq), and data analysis.....	2
Two-tailed RT-qPCR.....	3
Problem formulation	4
Materials and methods	4
Ethical consideration.....	4
Plasma collection and storage	5
Study outline	5
Total RNA extraction	8
Hand-On-Time (HOT) and Turn-Around-Time (TAT).....	9
QC assessment.....	9
Two-tailed RT-qPCR.....	9
Data analysis	10
Absolute quantification	10
Test subject: miR-223.....	10
Results	10
Time management and QC comparison between manual – and robotic extractions	10
Two-tailed RT-qPCR.....	11
Discussion	17
The total RNA extraction.....	17
QC assessment.....	18
Hands-On-Time (HOT) and Turn-Around-Time (TAT).....	20
Two-tailed RT-qPCR.....	21
Limitations of the study.....	23
Ethical aspect and societal impact of this study.....	23
Conclusion.....	24
Acknowledgement.....	25
References.....	26
Appendix 1	31
Appendix 2	32

Introduction

Sepsis is a frequently occurring cause of death among patients in hospitals worldwide. Time is of the essence for healthcare workers when carrying out point-of-care testing (POCT) to establish a diagnosis and treatment as quickly as possible. The complication stems from several comorbidities and primary diseases the patients have (Vincent et al., 2009). The definition of Sepsis has been changed over the years in large part because of the disproportionate focus on inflammation, or systemic inflammatory response syndrome (SIRS), which paints a misguided picture of the syndrome. The current definition of Sepsis, established in 2016, also called Sepsis-3, states that it is a life-threatening organ dysfunction caused by a dysregulated host response to an infection (Singer et al., 2016). In 2013 the expenses of Sepsis in the USA exceeded \$24 billion, which is approximately 13% of the US total hospital expenses. Interestingly, in the US in 2016, sepsis hospital stays accounted for only 3.6% of the total hospital stays (Torio et al., 2016). Today, the number is believed to have doubled (Centers for Medicare & Medicaid Services, 2023).

The knowledge of the diminished cognitive ability of Sepsis survivors' post-sepsis is increasingly revealed and presents even further societal resources and challenges (Iwashyna et al., 2010). However, the real impact of Sepsis may be exponentially higher due to the difficulties in laying the diagnosis. According to a population-based study in Sweden, by Ljungström et al (2019), the Sepsis incidences with fatal outcomes seem to increase with age. Bloodstream infections, like Sepsis (even though only one symptom of the condition), are believed to have a mortality rate of 30-50% worldwide, and the incidents increase at a rapid rate (1177 Vårdguiden, 2023). The course of events is unpredictably fast and may go from stable to life-threatening in a couple of hours. The common symptoms of patients are fever, muscle ache, diarrhea, vomiting, and shortness of breath (1177 Vårdguiden, 2023). Due to the fast course of events and limited knowledge of causing agents, the treatment of Sepsis is insufficient, and methods for detecting early-onset Sepsis (Bacteremia) and multiple biomarkers need to be established. At this moment, blood culturing is the current gold standard method of detecting Bacteremia. This presents a major challenge because it is time-consuming and in sepsis, time is scarce, and blood culturing may take up to 72 hours while life-threatening conditions can develop only after a couple of hours (Ljungström et al, 2019). Every hour of delayed use of effective antibiotic treatment reduces survival by 7.6% (Kumar et al., 2006).

Biomarkers are a set of indicators that can be measured and evaluate biological status in healthy and pathogenic processes (Androvic et al., 2017). There are multiple challenges when finding solid biomarkers, for instance, they should have enough specificity and sensitivity. At present, researchers are trying to establish a multiple biomarker panel with the idea that these would create a clearer and more precise diagnosis of Sepsis (Androvic et al., 2017). Today, diagnosing sepsis typically involves testing for individual biomarkers, such as C-reactive protein (CRP) or procalcitonin (PCT). However, these markers are not highly specific. Consequently, blood culturing remains the gold standard in clinical settings for identifying the causative agents. The most critical part in the case of sepsis is to treat septic patients with the correct antibiotics. A more accurate identification of the bacterium would not only save more lives but would also combat the antibiotic resistance we are facing today (Liu et al., 2018). A retrospective cohort study conducted by Kumar et al. (2006), showed that only 50% of patients received proper antibiotics within the first 6 hours of ongoing Sepsis. As previously stated, time is precious since every hour of delayed use of effective antibiotic treatment reduces survival by 7.6% (Kumar et al., 2006).

MicroRNA as biomarker

In more recent years, microRNA (miRNA) has established itself as a biomarker with great potential for many different diseases including cancer, HIV, Type-2 diabetes (T2D), and now also Sepsis (Formosa et al., 2022). In 2007 a study showed the manipulation of miR-125b and miR-155 could potentially facilitate the regulation of the response to endotoxin shock (Tili et al., 2007).

Furthermore, a paper published in 2009 suggested that miR-150 was a reliable prognostic marker for Sepsis (Vasilescu et al., 2009). MicroRNA is a small (approximately 22 nucleotides) non-coding molecule that plays a key role in the regulation of gene expression in eukaryotic cells (Lee et al., 1993). These small RNA molecules bind to target RNA and slice the mRNA, making it repress the translational process. MicroRNAs derive from two precursors. First, the Pri-miRNA (Primary-miRNA) in the nucleus later gets cleaved by Drosha (Ribonuclease) which gives rise to the second precursor Pre-miRNA (Pre-miRNA). MicroRNAs are mainly intracellular and travel back and forth in the cytoplasm and nucleus (approximately 75%) (Gagnon et al., 2014). However, they are also found in extracellular space where they display a hormone-like ability to travel from one cell to the next and bind to the cell-surface receptors (e.g. toll-like receptors) (Zhang et al., 2015). Furthermore, microRNA dysregulation has been proven to be an indicator of compromised immunogenic response thus making the colonization of the host easier for the intruder. This makes microRNA a promising diagnostic and/or prognostic device (Antonakos et al., 2022). Furthermore, miRNA has been shown in multiple studies to be more stable in fluids, this makes it very common to extract circulating miRNA from body fluids, such as blood and serum samples (Ho et al., 2022). However, everything has its pros and cons, and this small RNA is no exception. miRNA is in low amounts in the body and challenging to detect, coercing compensation with the extraction of higher volumes from patients is needed, which may not be optimal for an already compromised individual (Rogoete et al., 2018).

Measurement of miRNA expression

There are three major methods of measuring miRNA expression, that are used worldwide: Next-generation sequencing, microarray methods, and Reverse Transcription quantitative PCR (RT-qPCR) (Androvic et al., 2017). Microarray methods are expensive and not very specific; however, they can be used when observing multiple targets. RNA sequencing is implemented when discovering new miRNA. Nonetheless, the method is costly, has an undesirable workflow, and requires demanding data analysis. The RT-qPCR is popular when validating results; in other words, accuracy is paramount when you know what to look for. In addition, the sample size should be rather small for this method (Androvic et al., 2017). The RT-qPCR is the method of choice in terms of miRNA quantification (Krepelkova et al., 2009). However, this brilliant technique does not eliminate RNA's naturally perplexed biochemical properties. This includes properties such as the short length of miRNA (Krepelkova et al., 2019), and the discrimination between the two miRNA precursors and mature miRNA (Git et al., 2010; Willenbrock et al., 2009). In preparation for the PCR amplification the RNA (in this case miRNA) undergoes the reverse transcription transformation to the more stable form of double-stranded cDNA, which is recognized as the most delicate step in the process. Subsequently, the PCR reaction is performed to generate amplicons of the target, miRNA (Krepelkova et al., 2019).

qPCR, Quantification cycle (Cq), and data analysis

Quantitative polymerase chain reaction (qPCR) provides a sensitivity that makes it possible to quantify miRNA levels (Zampetaki & Mayr, 2012). The amplification curve generated during the qPCR run enables the researcher to analyze the Cq (Quantification cycle) values, and with the Cq value the N_0 (Starting concentration) of the target could be estimated (de Ronde et al., 2017). However, before using the data collected (Cq) the validity must be contemplated (e.g. check for off-target amplification). This can be done with a melt curve analysis, or another option is gel electrophoresis. In the melt curve analysis, temperatures correspond to a cDNA product range from 80-85 °C (Chan et al., 2023). Furthermore, assessment of the amplification curves is essential, which should contain an exponential phase and a plateau phase, if either is missing, it is most likely that the concentration of the template is below the qPCR detection limit (Kroh et al., 2010). Moreover, it is commonly used in triplicates (Technical replicates) when loading the qPCR plate, which provides aid for the precision of the qPCR (improvement of experimental variation, detection of outliers, etc). Consequently, the mean of the replicates is then used to calculate N_0 . As

a rule of thumb, the replicates should not be ≥ 0.5 cycles apart, if that is the case, the sample ought to be categorized as missing data. Nonetheless, it is common to get amplification on only two of the three triplicates, in that case, the two former ones are likely true positives, and the third (Without Cq) technical error. Furthermore, Cq-values ≥ 35 ought to be removed as well, because these are most likely non-informative amplifications. Due to the naturally low quantities of circulating miRNA, it should be expected a lot of missing data (e.g. no amplification), and thus statistical power (McCall et al., 2014). Moreover, a major challenge when working with miRNA is to also be able to distinguish between the different kinds of missing data such as off-targets, too wide of a gap between the replicates, and unanalyzable amplification curves (de Ronde, et al 2017). Therefore, the reproducibility of work with miRNA is very difficult. To date, there is no consensus in the scientific literature on how to handle and interpret the Cq-values generated from qPCR (de Ronde et al, 2017).

Furthermore, it has been reported a great inconsistency in miRNA analysis from the same sample depending on which extracting kit is used (Fauth et al., 2019). This was demonstrated by Brunet-Vega et al., (2015), where miRNA was recovered from the same sample plasma using five different miRNA extraction kits. As mentioned above, RNA extraction is the most critical step in the process. Due to the unstable form of RNA, it is impossible to avoid degradation during extraction. As reported by Ibberson et al., (2009), the degradation of the total RNA, jeopardizes the predictability of measuring miRNA expression. Therefore, measuring the RNA quality is a standard procedure after extraction. This is done with the measurements of OD_{260/280} and OD_{260/230}, employing photometrical methods. However, photometrical methods do not distinguish between different RNA species, thus the concentration of miRNA cannot be analyzed, but the total RNA concentration. Nevertheless, there are some instruments available that are capable of measuring miRNA absolute amount (pg/ μ l), for instance, the Agilent small RNA assay, which provides both numerical results and “gel-like images” (Fleige et al., 2006).

In qPCR, endogenous controls (untreated samples) are used to enhance the validity of the results. These controls serve as a reference point (baseline) for the treated samples, enabling data normalization and more accurate quantification. This approach allows for the analysis of differences in Cq-values between treated and untreated samples. (Radonić et al., 2004).

In absolute quantification, a standard curve (Serial dilution) is required to quantify the target (Nucleic acids) in the samples. It is also paramount for the validation of the qPCR reaction, e.g. the efficiency. A reliable standard curve should have an efficiency (%) between 90-110% and an R² (Pearson's correlation efficiency) of at least 0.98, and with a slope between -3.1 - -3.5. If these criteria are not met, the accuracy of the calculations will be incorrect, and thus the absolute quantification of the samples will be false. An equation (Quantity = $10^{(Cq - b/m)}$) will be generated from the SC, and the Cq of samples retrieved from the qPCR reaction will be incorporated into the equation (Iguchi et al., 2018).

Two-tailed RT-qPCR

Inherently, the small size of miRNA (18-24 nt) makes them challenging to detect with qPCR, and the close family members of the small RNA with similar sequences makes it hard to discriminate. The two-tailed RT-qPCR is a two-step method that Androvic et al (2017) developed to combat these issues. The Two-tailed RT primers are equipped with Hemiprobes connected by a hairpin structure. Together, one at each end, bind to the target (miRNA), which forms a stable complex. Furthermore, the RT enzyme binds to the 3'-end of the hybridized Two-tailed RT primer, causing elongation and “tailed-cDNA”. Consequently, the cDNA is amplified with qPCR with target-specific primers. This promising method allows POCT with analysis <2.5 hours.

Problem formulation

Sepsis remains a major global health problem, it is estimated that ~33 million people are affected each year, and the mortality rates are painfully high (Ljungström et al., 2019). Today, the diagnostics of sepsis provided, are inadequate, in terms of both precision and speed a diagnosis could be carried out. Early detection of sepsis requires multiplex biomarkers (5-15) as opposed to singleplex. Furthermore, each biomarker must have the specificity to give the accurate assessment, today the standard biomarkers (C-reactive protein etc) are not very specific, in fact, not at all (Ljungström et al., 2017).

Even if miRNA shows great potential as a biomarker and could be included in a multiplex panel of biomarkers, that has been developed by Ljungström et al (2017). The challenging task of quantification and reproducibility of miRNA research is one major obstacle. As mentioned previously, there is no consensus in the scientific literature on how to generate the data (Laboratory procedure) and also how to interpret the generated data (de Ronde et al., 2017). The hospitals are in dire need for a much more efficient methodology to give the septic patients the necessary treatment. However, the research is in full bloom and the future looks promising in the field of molecular diagnostics for sepsis.

Aim

This project is a continuation of the “Future Diagnostics of Sepsis” study, conducted at the University of Skövde. As the name of the project implies, the goal of the study is to improve the diagnostics of Sepsis in terms of earlier detection and with higher precision. Even though this experiment has been carried out multiple times before, the contribution of this work is to supply more data, and thus evidence, for one (e.g. miR-Seps 7) of many candidates investigated in this larger project.

This project had two primary aims: first, to determine whether the miRNA candidate (miRSeps-7), recovered from healthy human plasma, could be detected and quantified (Absolute quantification) using the Two-tailed RT-qPCR method; and second, to compare the efficiency of two total RNA extraction methods (manual and robotic (Qiacube) extraction). This comparison was based on measuring the Hands-On Time (HOT) and Turnaround Time (TAT) during the extraction process, along with analyzing the Cq values obtained from the qPCR reactions. Additionally, QC check was performed post RNA extraction.

The research question of this study is a two-part question is it more effective to extract RNA manually or robotically? Effectiveness is defined as how fast (in minutes) the process is, and more importantly with what degree of accuracy (Cq-value) it can be performed. Secondly, is the candidate detectable and quantifiable when using the two-tailed RT-qPCR technique with the implementation of the absolute quantification method?

Materials and methods

Ethical consideration

The blood samples used in this study came from healthy (e.g. no sepsis-associated illness) volunteers who had been informed about the study beforehand. The project “The future diagnostics of Sepsis”, which this study is an incremental part of, was approved in 2011 by The Regional Ethics Committee in Gothenburg (no. 376-2011) and thus qualifies as an ethical study that intends to result in a more effective diagnosis of Sepsis which will allow healthcare workers to save more lives.

Plasma collection and storage

The plasma was obtained from the blood of 8 non-septic donors and stored in citrate tubes to prevent coagulation. It was kept in a -80 °C freezer in the laboratory at the University of Skövde. Upon total RNA extraction, the samples were centrifuged at 8,000 rcf for 5 minutes.

Study outline

In this study, total RNA was extracted from 20 plasma samples obtained from non-septic individuals (n=8) (Figure 1.). The extractions were evenly divided between manual methods (n=10) and robotic methods using Qiacube (n=10). Among these, 12 samples were spiked with synthetic miRSeps-7, while 8 were not (Untreated). Each sample was labeled with a number (1-9) corresponding to the individual, followed by indications of whether it was extracted manually (M) or robotically (R), and whether it was spiked (S) or non-spiked (NS). For example, the label "1MNS" denotes the sample from individual 1, extracted manually and not treated with spike-in. For every tube of plasma (Individual), one manual - and one robotic extraction were performed simultaneously to ensure that the samples were paired. This was done to facilitate a direct comparison between the two extraction methods (Figure 2.). Hands-On-Time (HOT) and Turn-around-Time were recorded, and QC check was performed. Followed by the Two-tailed RT-qPCR method (Figure 3.). Finally, a statistical analysis where the unusable data were filtered out, a comparison between the two total RNA extraction methods was made, and absolute quantification was performed (Figure 4.).

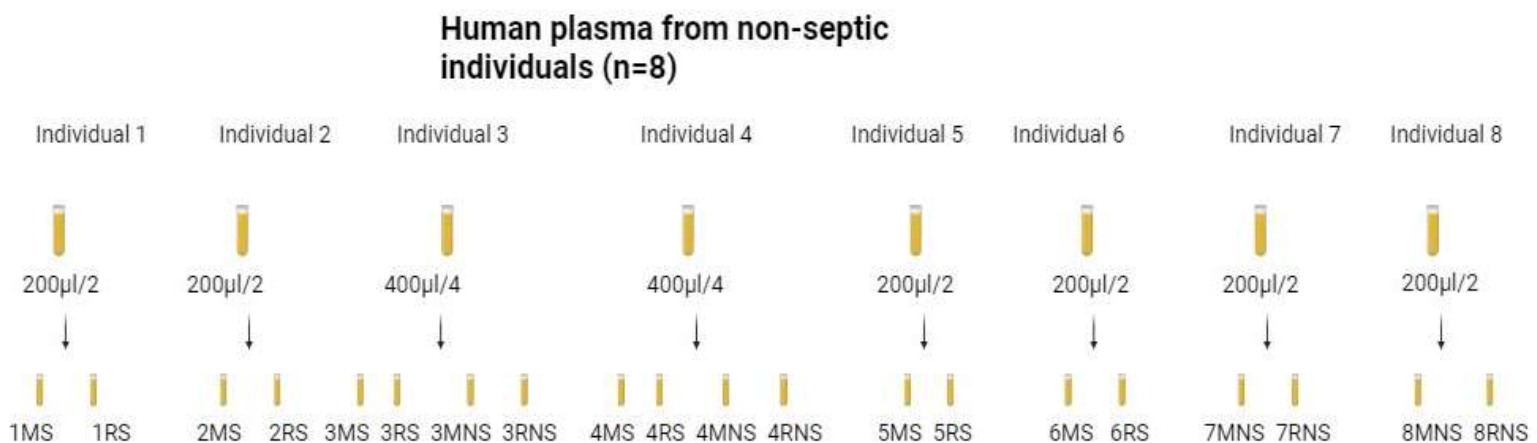


Figure 1. Schematic representation of the samples (n=20) collected from frozen (-80°C) plasma tubes (n=8), where each tube contains plasma from one individual. Tubes from individuals 1, 2, 5, 6, 7, 8, contained 200µl plasma and were divided into two, to ensure 100µl for one manual total RNA extraction and one robotic (Qubit) total RNA extraction. Tube from individuals 3 & 4 contained 400µl which was divided by four to facilitate four samples, including both spiked - and non-spiked samples.

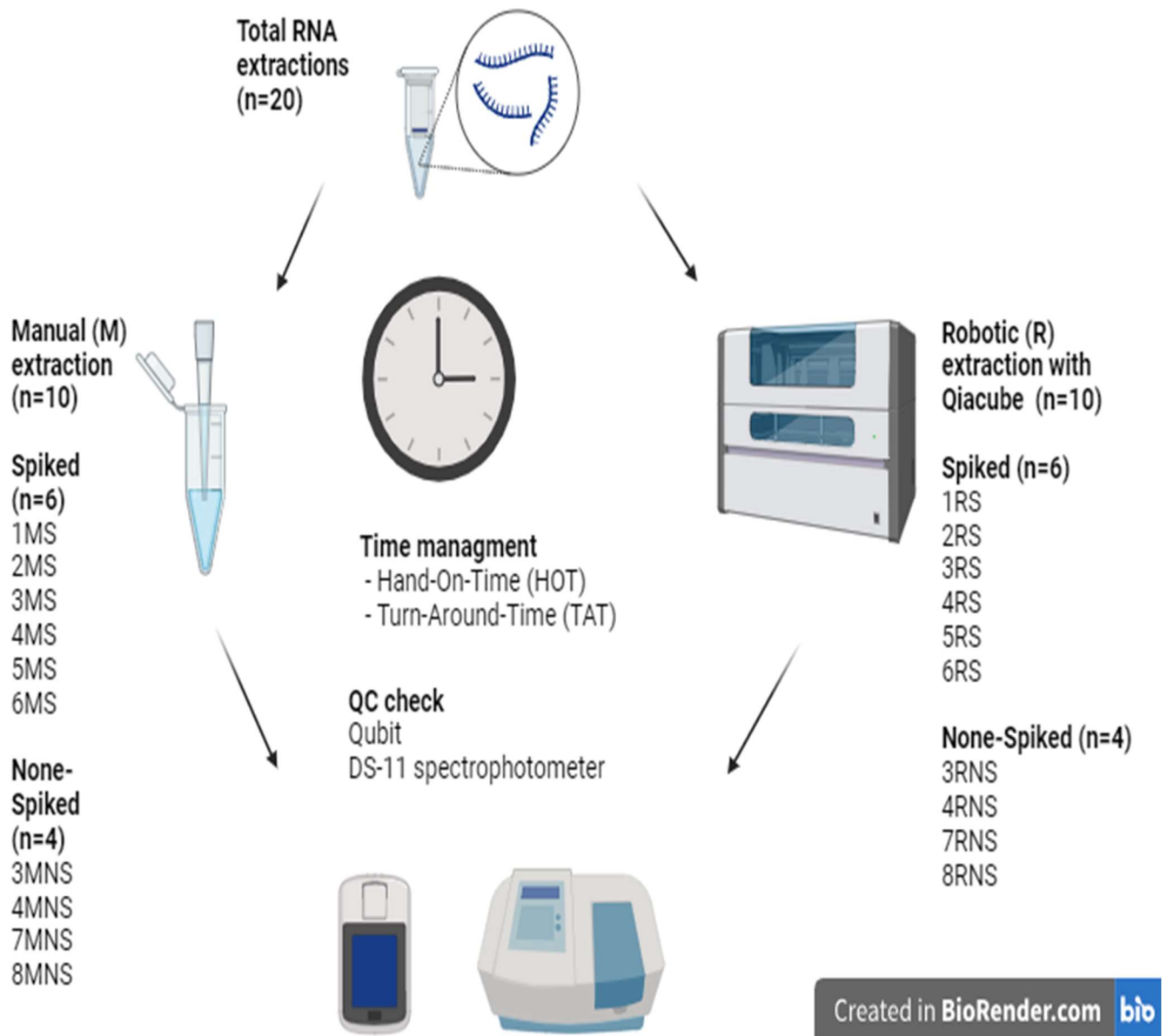


Figure 2. Workflow of the total RNA extraction with both spiked – and non-spiked samples. The extractions were carried out manually – and robotically while HOT and TAT were recorded. Post extractions, QC check was performed with Qubit and DS-11 spectrophotometer.

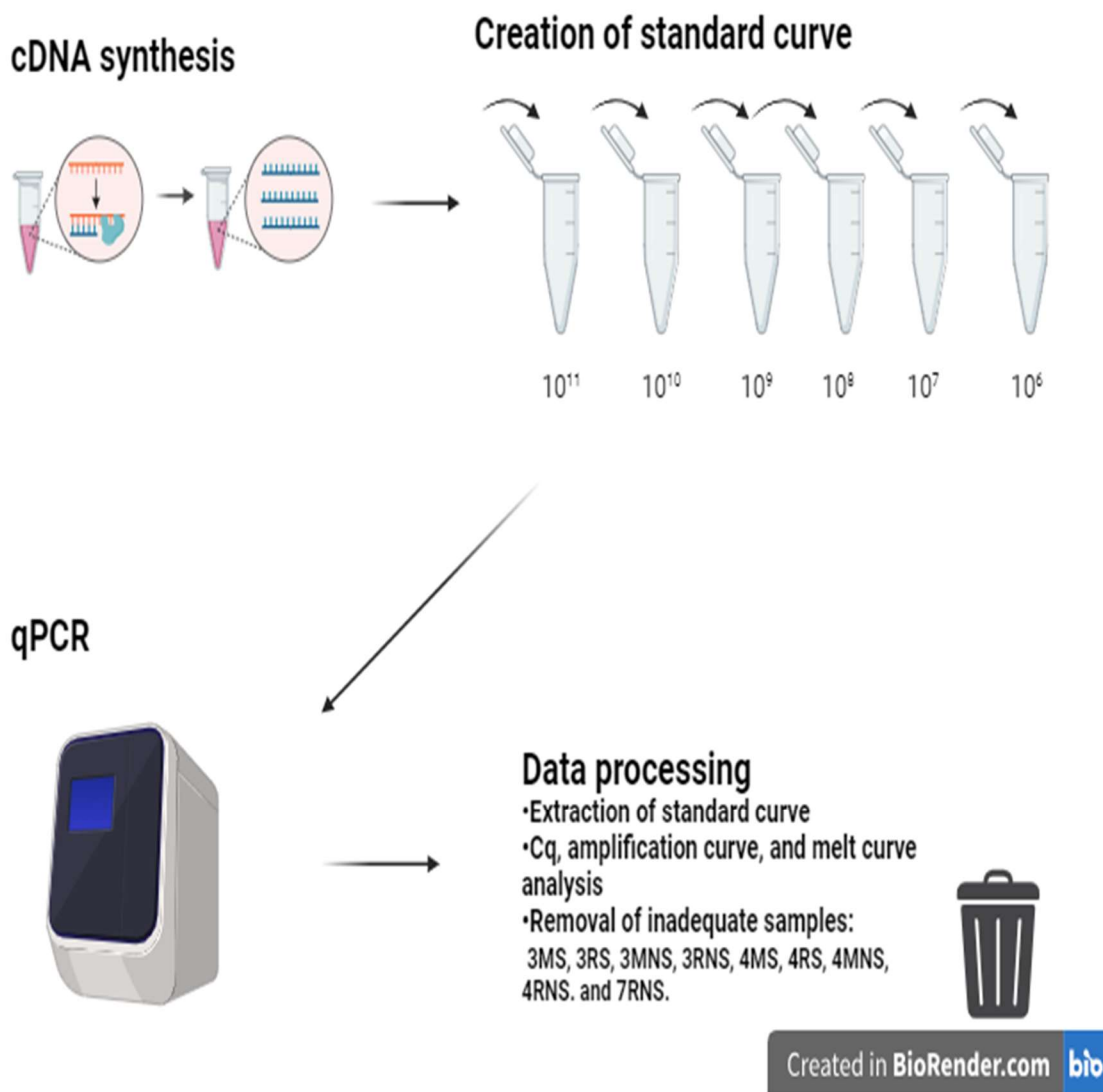


Figure 3. Workflow of the Two-tailed RT-qPCR. miRNA was converted into cDNA with two-tailed RT primers followed by six-point 10-fold serial dilution of synthetic miRseps-7 (Stock solution with copy number 10^{11}). Subsequently, qPCR was performed where data were collected and processed, including amplification – and melt curve analyses.

Statistical analysis (IBM SPSS Statistics, V-27)
 • Manual extraction vs Robotic (Qiacube) extraction
 - HOT & TAT, and QC comparison



Absolute quantification ($10^{(Cq-b/m)}$)
 1MS
 1RS
 2MS
 2RS
 3MS
 3RS
 6RS
 7MNS
 8MNS
 8RNS

Created in BioRender.com

Figure 4. Workflow of the statistical analysis, comparison of the two extraction methods, and absolute quantification. The absolute quantification could be performed with the linear equation generated from the standard curve ($10^6 - 10^{11}$) created with synthesis miRSeps-7.

Total RNA extraction

Manual extractions

100 μ l plasma was used instead of the 200 μ l (recommended by the kits' manufacturer) due to the limited starting material available. All components were cut in half (Table 1.). The plasma was thawed and centrifuged for 5 minutes before 100 μ l of plasma was collected. For each extraction, two paired 100 μ l (each) plasma samples were extracted at four different occasions. Half of the samples were spiked (treated), with synthetic miRSeps-7 (3.5×10^6 copies) according to the protocol miRNeasy Serum/Plasma Advanced Handbook, 2021 (Qiagen), Protocol: Purification of total RNA, including miRNA, From Serum and Plasma.

Table 1. Components and volumes of the manual total RNA extractions

Components	Volume (μ l)
Buffer RPL	30
Buffer RPP	30
Buffer RWT	350
Buffer RPE	250
Ethanol (80%)	250
Nuclease-free water	14

Robotic extractions (Qiacube)

100 μ l plasma was used, like the manual extractions. The robotic extractions were carried out manually until step 6 according to the protocol miRNeasy Serum/Plasma Advanced Handbook, 2021 (Qiagen), Protocol: Purification of total RNA, including miRNA, From Serum and Plasma. However, due to the required volume for the Qiacube (200 μ l), 100 μ l extra nuclease-free water was added before the samples were transferred into the Qiacube. All remaining components were in the same volumes as the manual extractions (Table 1.).

Hand-On-Time (HOT) and Turn-Around-Time (TAT)

Time was recorded during the total RNA extraction processes. The data was collected and compared between the manual – and robotic extractions. The time was recorded from the start of the extraction process (100 µl plasma) until the 14µl eluates were collected. Hands-on-time was gathered by TAT (The whole duration of process) minus the inactive time (e.g. centrifugation etc). This was measured six times. The Mean and SD were calculated from both measurements and compared between the two extraction methods.

QC assessment

Following the time management, eluates were analyzed. The concentration and purity of the total RNA of the manual and robotic extractions were measured according to ng/µl and A_{260}/A_{280} ratios with a DS-11 spectrophotometer (DeNovix). 1 µl of analyte were loaded onto the apparatus. In addition, the quantity of small RNA extracts was measured by Qubit® 2.0 fluorometer (Thermofisher) according to the protocol Qubit® microRNA Assay Kits (Life Technologies).

Two-tailed RT-qPCR

After QC assessment, cDNA synthesis was performed to convert miRNA to cDNA with the guidance of the protocol for two-tailed priming of the GrandScript cDNA FreePrime kit (TATAA). For this reaction, miRSeps-7 Reverse Transcriptase primers (Integrated DNA Technologies) were used and the volume was adjusted according to the protocol (Table 2.). Apart from the eluates, the reaction included a 10-fold serial dilution with six points ($10^6 - 10^{11}$) taken from the stock solution (Given by supervisor) of the synthetic candidate miRSeps-7 with copy number 10^{12} , No-Reverse-Transcriptase (-RT) and No-Template (NTC). Furthermore, the samples were transferred to a thermocycler (Biometra) and incubated for 50 min at 42 °C, 5 min at 85 °C, and finally held at 4 °C for 20 min.

Table 2. Components of the Reverse-Transcription reaction.

Component	Volume (µl)
GrandScript FreePrime Reaction Mix (5x)	2.0
GrandScript RT Enzyme	0.5
RNA sample	4.0
Two-tailed primer (0.2µM)	2.5
GSP enhancer	1.0
In total	10.0

The newly synthesized cDNA samples underwent quantitative polymerase chain reaction (qPCR) performed with SYBR GrandMaster Mix (TATAA Biocenter) protocol. Due to limited materials, all the components in the reaction were reduced by half (Table 3.). In addition, normalizer dye (ROX) was added to the master mix before samples were pipetted in triplicates onto the 96-well plate. In addition, a No-amplification-Control (NAC) in triplicates was added to the reaction. In the reaction, the AriaMx Real-time PCR system (Agilent) was used and followed the cycling protocol of the hot start stage, 30 s at 95°C. Followed by amplification, 95°C for 5 s, subsequently by 60°C for 95 s, and lastly 72°C for 30 s. In addition, a melt curve was generated by cycling repeatedly to 95°C for 30 s, 60°C for 30 s, and again at 95°C for 30 s.

Table 3. Components of the qPCR reaction for one triplicate.

Components	Volume (µl)
TATAA SYBR® GrandMaster® Mix (2x)	5.0
Nuclease-free water*	2.4
Forward primer (0.2 µM)	0.2
Reverse primer (0.2 µM)	0.2
cDNA sample	2.0
Rox dye (50X)	0.2
In total	10.2

*The volume of nuclease-free water depended on which well. Wells containing the No-RT (-RT) and No-Amplification-Control (NAC) had 0.4 µl NFW instead of the primers. The No-Template-Control (NTC) had 2.0 µl NFW in replacement of the cDNA template

Data analysis

After completing all the two-tailed RT-qPCR reactions, data collection and calculation were performed using IBM SPSS Statistics (Version 27). Subsequently, an extensive analysis was conducted with the primary objective of comparing the two total RNA extraction methods. Initially, the mean and standard deviation (Mean ± SD) of HOT and TAT were compared between the two extraction methods. A Shapiro-Wilk test was utilized to assess normality, with the significance level set at $\alpha = 0.05$.

The Cq values underwent a selection process, excluding samples deemed as non-informative. Samples with Cq values ≥ 35 and differences in Cq ≥ 0.5 between technical replicates were discarded. Additionally, apart from the non-amplified samples, a melt curve analysis was performed to remove all off-targets (e.g., targets not within 80-85°C). Subsequently, the Cq values from the paired samples were compared between the manual – and robotic extractions.

Absolute quantification

The ten-fold diluted serial dilution with six points underwent the two-tailed RT-qPCR, which was extracted from the AriaMx software after the qPCR run and put in Windows Excel. The linear regression equation ($y=mx + b$) retrieved could be used to calculate the estimated concentrations of spiked – and non-spiked extracted samples. The absolute quantification was obtained with: N_n (Logarithmic initial copy number) = $10^{(n-b/m)}$ where $n=Cq$, and $m=slope \rightarrow Quantity = 10^{(Cq-b/m)}$.

Test subject: miR-223

To get to know and ensure that the methodology (Two-tailed RT-qPCR) worked, known synthetic miRNA (miR-223) was deliberately used as a test subject for all the procedures before the official project began with the candidate, miRSeps-7 (Results not disclosed).

Results

Time management and QC comparison between manual – and robotic extractions

Time estimation of total RNA extractions

To compare the efficacy of the two extraction methods, the Hands-On-Time (HOT) and Turn-Around-Time (TAT) were recorded for the manual extraction and the robotic extraction (Qiacube). Two extractions per method each time, in a total of six times, were recorded. There was no

significant difference between the two extraction methods ($p > 0.05$) for both HOT and TAT (Table 4.). The HOT favored robotic extractions ($19 < 24$) and TAT the manual method ($42 < 46$). Noteworthy, the standard deviation was considered greater for the manual extractions for both HOT and TAT. Indicating, a greater spread around the mean, and thus a more unpredictable time estimation. However, due to the small sample size, this cannot be interpreted as informative. In addition, the Qiacubes full capacity in terms of the number of samples that could be loaded onto the machine simultaneously, was not explored in the present study. The limitations of this comparative study will be further elaborated on in the discussion section.

Table 4. Illustrates the average time and standard deviation in minutes of Hands-On-Time (HOT) and Turn-Around-Time for manual and robotic total RNA extractions.

Extractions method	HOT (Mean±SD)	TAT (Mean±SD)
Manual (n=6)	24±6.19	42±8.52
Robotic (n=6)	19±2.31	46±5.13

QC assessment of total RNA extractions

All extractions had concentrations too low for detection by the Qubit 4 fluorometer (Thermo Fisher Scientific). The Qubit's detection limit for the miRNA assay kit was 0.5 ng/μl, which none of the eluates (n=20) reached, resulting in no detectable results.

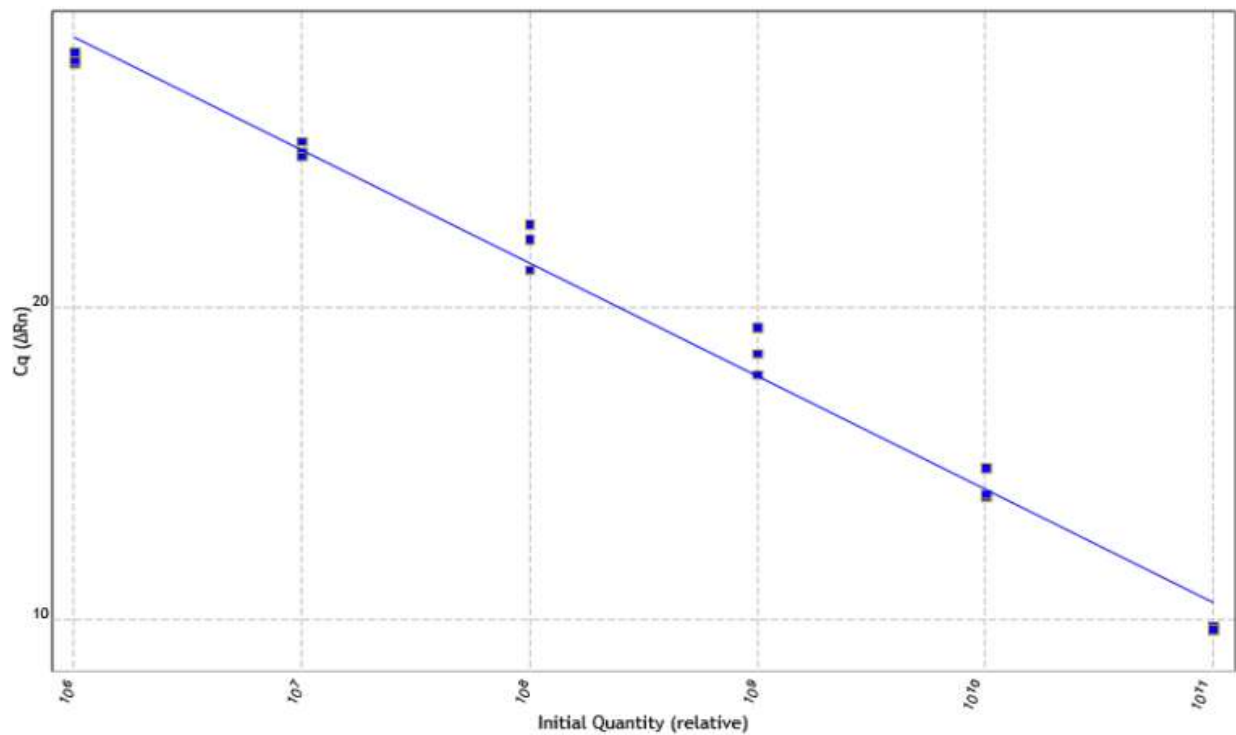
The concentration and A260/A280 absorption ratio of the total RNA extractions, both spiked and non-spiked, were measured using a DS-11 spectrophotometer (DeNovix), similarly to the Qubit, the concentrations (ng/μl) were too low, even though measurements were retrieved, they were too low to give any reliable data (Appendix 2, Table 1.), Analytes with concentrations <20ng/μl produce unreliable results and therefore ought not to be presented in experimental findings (Desjardins & Conklin, 2010). "

Two-tailed RT-qPCR

Standard curve with miRSeps-7

For absolute quantification, a standard curve with six points of synthetic miRSeps-7 was generated (copy number $10^6 - 10^{11}$) with the Two-tailed RT-qPCR method. Additionally, negative controls, Non-Amplification-Control (NAC), No-Reverse-Transcriptase (-RT), and No-Template-Control (NTC) were included. The standard curve had an efficiency of 0.90 and R^2 at 0.99, indicating a sufficient standard curve (Iguchi et al., 2018). Moreover, according to B) in Figure 5. there is one distinct peak (~ 80-85°C) displayed in the melt curve indicating the desired result with one product The standard curve plot (Figure 5., A) showcased that the blue dots aligned for the most part with the trendline (blue line).

A)



B)

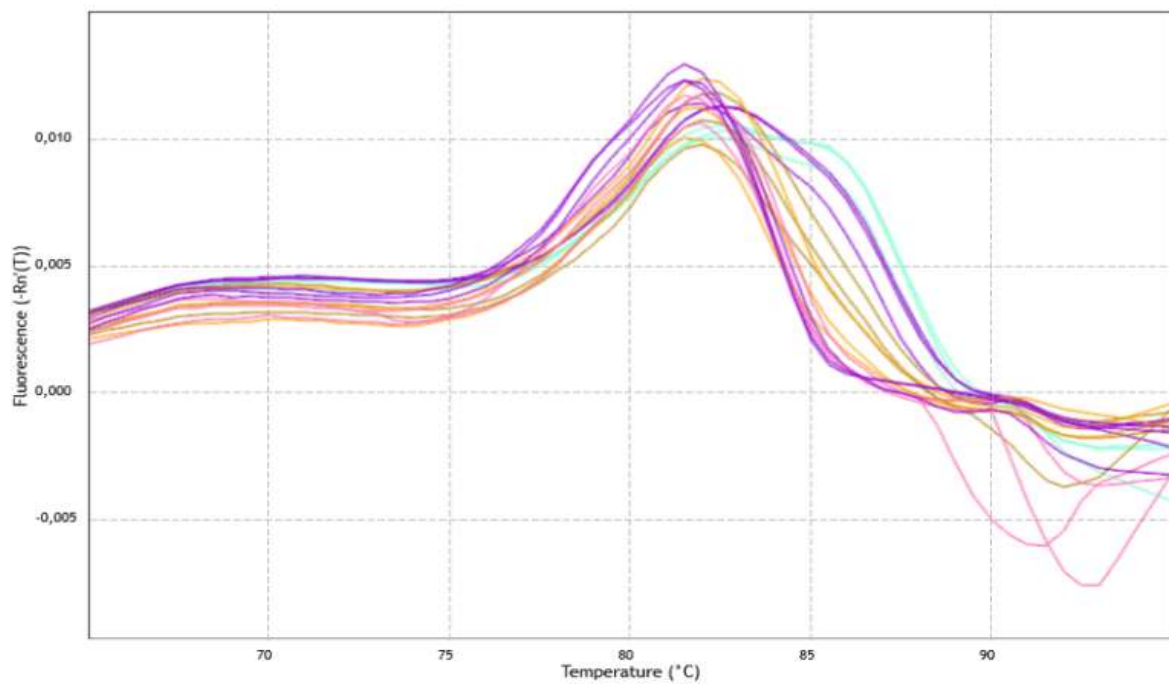


Figure 5. Showcase the graphical displays of the qPCR reaction with the standard curve. A) standard curve with Cq-values against copy number ($10^6 - 10^{11}$). Efficiency (%) of 0.90 the slope of -3.591, and R^2 of 0.99. Cq(ΔRn) on the y-axis and Initial Quantity (Relative) on the x-axis. B) Melt curve with fluorescent (y-axis) compared to temperature °C. (x-axis) Fluorescent threshold: 0.05 (ΔRn).

Individual Cq-values of the spiked – and non-spiked extracted samples

The average Cq-value of replicates was obtained from each sample with spiked samples (Table 5.) and non-spiked samples (Table 6.). Individuals 3 and 4 appear in both tables (Tables 5 & 6.). The 4RS sample was dismissed due to off-target amplification. This is illustrated in the melt curve graph from the qPCR reaction (Figure 6.), where the products from the 2/3 replicates are outside of the wanted product melting temperature (80-85°C). The off-target amplification appeared for samples 3MNS (Figure 8.) and 7RNS (Figure 7.) as well, where only 1/3 replicate for both samples (3MNS & 7RNS) was on-target. However, 7RNS was discarded due to Cq >35 (38.3). Noteworthy, triplicates of 3MNS (Figure 8.) display multiple peaks where the first one (for all three), began at ~77°C, which could be an indication of contamination (Chan et al., 2023).

Furthermore, none of the remaining samples, which were discarded due to unusable Cq values (off-target amplification), exceeded the threshold of coefficient variance ($4\% \geq$), which indicates that the technical variability was sufficient and thus repeatable. The coefficient variance (CV) is the size of the standard deviation in contrast to its mean (SD/Mean), in other words, the higher the CV is, the greater the spread is around the mean (Mestdagh et al., 2008).

Table 5. Mean and standard deviation of Cq-value for spiked samples of manual – and robotic extractions.

INDIVIDUALS	MANUAL (AVG±SD)	ROBOTIC (AVG±SD)
1	24.17±0.30	25.33±0.25
2	24.5±0.08	23.9±0.11
3	26.59±0.14 (T)*	24.9±0.23
4	-*	38.3±0.02*
5	-*	-*
6	-*	26.8±0.04 (T)*

*No Cq-values detected

*(T)=Triplicates

*Discarded (Non-informative)

Table 6. Mean and standard deviation of Cq-value for spiked samples of manual – and robotic extractions.

INDIVIDUALS	MANUAL (AVG±SD)	ROBOTIC (AVG±SD)
3	32.71±0.15*	-*
4	-*	-*
7	26.07±0.00	31.7±0.19*
8	26.41±0.17 (T)*	26.43±0.17

*No Cq-values detected

*(T)=Triplicates

*Discarded (Off-targets)

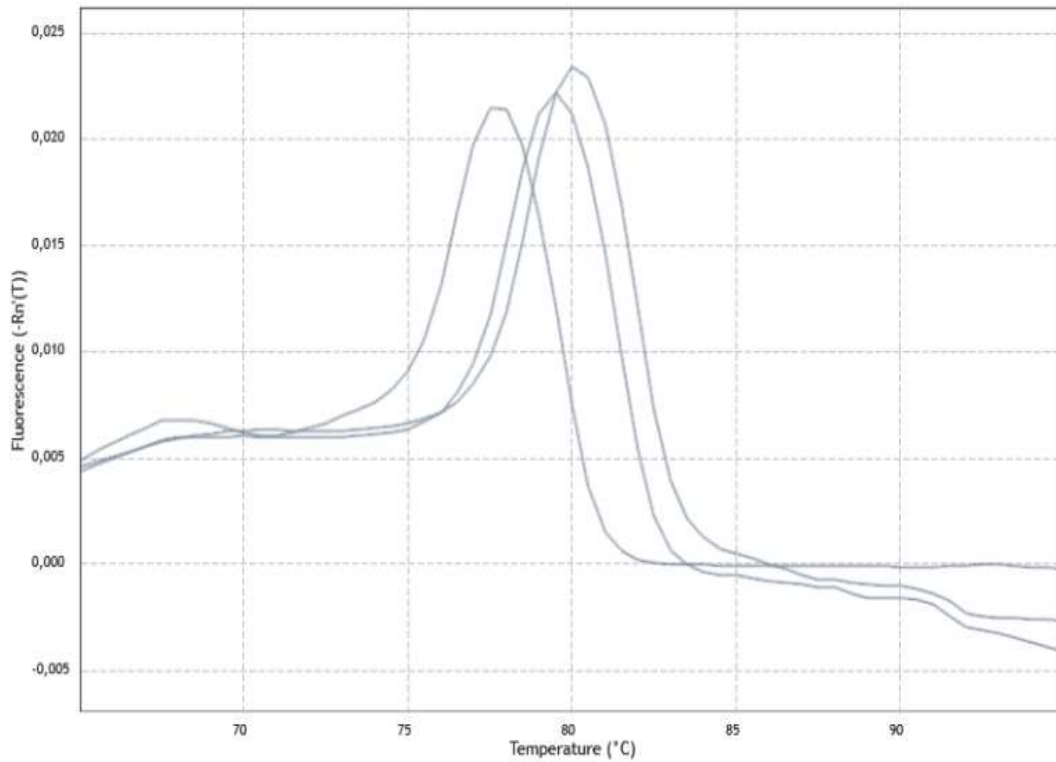


Figure 6. Depicts melt curve graph (qPCR) of triplicates from samples 4RS. The fluorescence light on the y-axis and the temperature (°C) on the x-axis. Only one out of the three had a melting temperature inside of the acceptable range (80-85°C). The other two with melting temperatures in the range of 77-79°C (e.g. off-targets).

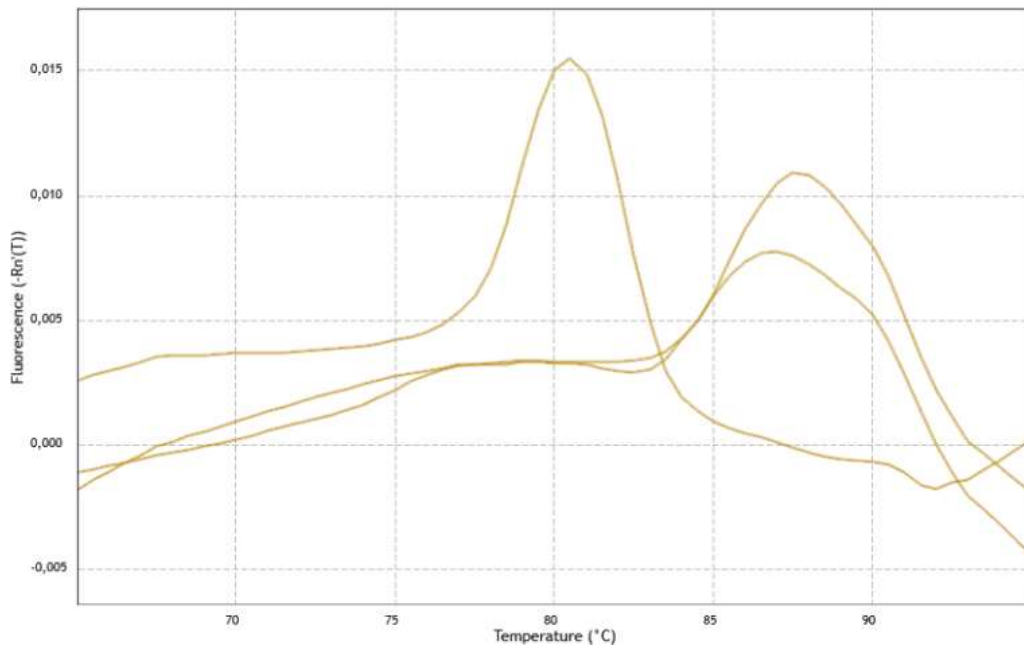


Figure 7. Depicts melt curve graph (qPCR) of triplicates of sample 7RNS. The fluorescence light on the y-axis and the temperature (°C) on the x-axis. Only one of the three had a melting temperature inside the acceptable range (80-85°C). Products with melting temperatures (°C) of 80.5, 87.0, and 87.5.

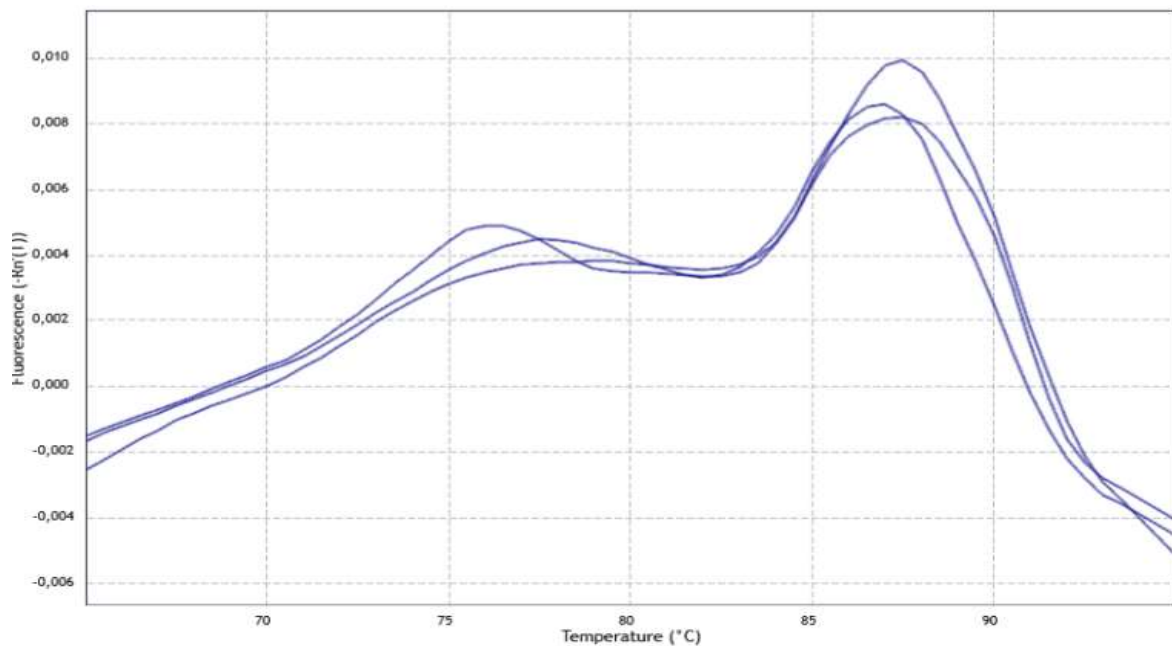


Figure 8. Depicts melt curve graph (qPCR) of triplicates of samples 3MNS. The fluorescence light on the y-axis and the temperature (°C) on the x-axis. All three triplicates with melting product of 87.0 – 87.5 (Off-targets).

Comparison of Cq values between extraction methods

After the unusable samples were discarded, the Cq values from the paired samples were compared between the two extraction methods. This comparison was analyzed on four individuals (1,2,3, and 8). The standard deviation was acquired to get the coefficient variance ($SD/Mean = CV$) which is a measurement of repeatability. The common threshold set for CV ranges from 1-4%, meaning that $CV >4\%$ is unreliable (Nettle et al., 2019). In this case, none of the CVs for the technical replicates exceeded 4%, and only samples from individual 1 (1MS & 1RS) were $\geq 1\%$, at 1.2%(1MS) and 1.0%(1RS) (Table 4.). A $CV \geq 4\%$ is an indication of a compromised qPCR assay, errors regarding contamination of reagent, pipetting, and fluctuation in temperature during the reaction (Ban & Joo Song, 2022). The results (Table 4.) showcase low variability, thus reliable system precision. In addition, the low SE (Standard Error) displayed across all samples is another measurement of solid repeatability, e.g. low variability between technical replicates. However, it is important to take the small sample size among the replicates into consideration, since this may lead to false reflection of the true variability (Ban & Joo Song, 2022).

The differences in the means between the two extraction methods are not statistically significant. Notably, in one of the four paired samples (Individual 8), no difference was observed. The largest difference was observed in Individual 1, where the 1RS outperformed the 1MS by 1.16 cycles (Table 4). However, the small sample size precludes drawing any definitive conclusions from these results.

Table 4. Comparison of mean and differences of the Cq values between the two extraction groups

INDIVIDUALS	MANUAL (AVG±SD)	CV	SE	ROBOTIC (AVG±SD)	CV	SE	DIFFERENCE (AVG)
1 (S)*	24.17±0.30	1.2%	0.21	25.33±0.25	1.0%	0.18	-1.16
2 (S)*	24.5±0.08	0.3%	0.06	23.9±0.11	0.5%	0.08	0.6
3 (S)*	26.59±0.14	0.5%	0.08	24.9±0.23	0.9%	0.13	1.69
8 (NS)*	(T)* 26.41±0.17	0.6%	0.10	(T)* 26.41±0.17	0.6%	0.10	0.00

*No Cq-values detected

*(T)=Triplicates

*(S) = Spiked

*(NS) = Non-spiked

Absolute quantification of miRSepts-7 from spiked – and non-spiked extractions

The standard curve generated had an efficiency of 90%. and the Pearson's coefficient (R^2) was at 0.9939 (Table 5.), demonstrating the linearity in the trendline. The absolute quantification was calculated on manual (n=5) – and robotic (n=5) extractions of cDNA samples including both spiked – and non-spiked duplicates (Table 6.). The equation from the trendline of the standard curve was retrieved and used to calculate the absolute quantification: $y = -3.591\ln(x) + 47,14$ (Figure 9.)

Table 5. qPCR amplification efficiency of the standard curve.

Variable	Value
Slope (m)	-3.591
In equation for E	E = 1.899
In equation for E%	E% = 90%

*Calculation: $E = 10^{(-1/m)} \rightarrow$ conversion to percentage $E\% = (E - 1) \times 100$.

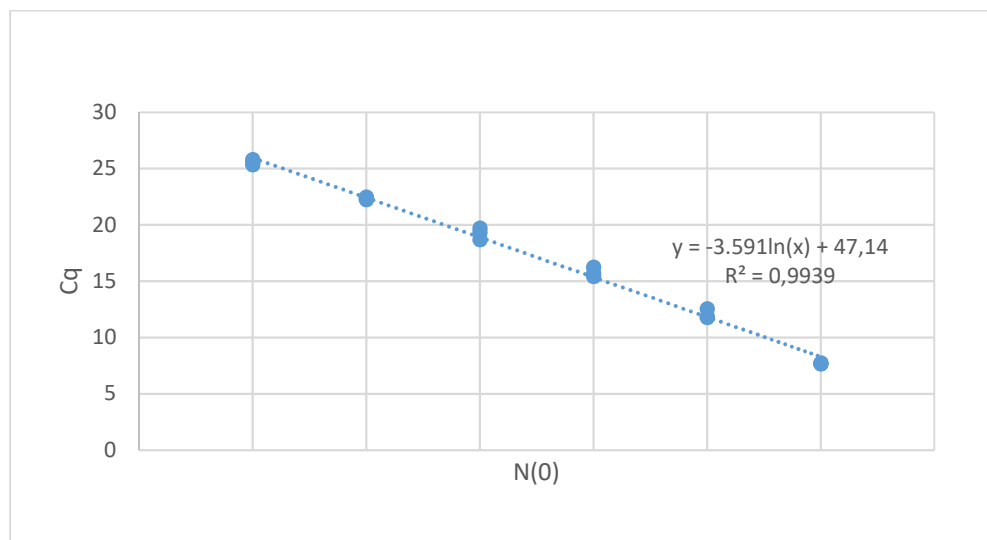


Figure 9. Depicts the standard curve obtained from qPCR reaction with miRSepts-7 (copy number $10^6 - 10^{11}$). Cq(ΔRn) on the y-axis and Initial Quantity (Relative) on the x-axis.

The equation gathered from the standard curve was used in the following equation: Quantity = $10^{(Cq - b/m)}$ where the mean of Cq-values was taken and applied to the equation.

In total, 10 samples were subjected to absolute quantification (Table 6.). The average Cq values of the replicates were converted into logarithmic numbers with the linear regression equation extracted from the standard curve (Figure 9.). The spiked samples (n=7) act as positive controls,

to facilitate the accuracy and reproducibility of the results (Want et al., 2023). The spike-ins ($3.5\mu\text{l} \times 10^6$ copies) were aliquoted to plasma samples ($n=12$) during the total RNA extraction process. Consequently, a theoretical quantity was established for the spike-ins ($3.5\mu\text{l} \times 10^5/14\mu\text{l} = 25000$ copies/ μl). However, only 7/12 of the spiked samples made it for quantification. As depicted (Table 6.), the spiked (S) samples ($n=7$) have the log copy number ranging from the lowest at $10^{5.72}$ (3MS) to the highest at $10^{6.47}$ (2RS). Whereas, among the none-spiked (NS) samples 3/8 could be quantified with the lowest copy number of $10^{5.76}$ (8RNS) and the highest copy number of $10^{5.86}$ (7MNS). Therefore, the results showcase a clear trend that the treated (spiked samples) have on average higher copy numbers of miRSeps-7 than the untreated (None-spiked samples), which is an indicator of on-target amplification (Androvic et al., 2017), since the higher the Cq-value the lower the copy numbers. However, since no absolute quantification could be performed on both spiked – and non-spiked samples from the same individuals, these numbers cannot be the basis for any calculations (e.g. Treated – Untreated = Natural amount of circulating miRSeps-7), since the potential vast variability of the amount of miRSeps-7 between individuals ought to be considered (El-Khoury et al., 2016).

Table 6. Depicts Cq-values, log copy numbers, and copies/ $2\mu\text{l}$ of 10/20 samples.

Samples	Cq-value (Avg\pmSD)	Log copy number	Copies/$2\mu\text{l}$
1MS	24.17 \pm 0.30	$10^{6.39}$	1996262
1RS	25.33 \pm 0.25	$10^{6.07}$	1174897
2MS	24.5 \pm 0.08	$10^{6.29}$	1949844
2RS	23.9 \pm 0.11	$10^{6.47}$	2951209
3MS	26.59 \pm 0.14 (T)*	$10^{5.72}$	524807
3RS	24.9 \pm 0.23	$10^{6.19}$	1548816
6RS	26.8 \pm 0.04 (T)*	$10^{5.66}$	457088
7MNS	26.07 \pm 0.00	$10^{5.86}$	724435
8MNS	26.41 \pm 0.17 (T)*	$10^{5.77}$	588843
8RNS	26.43 \pm 0.17	$10^{5.76}$	575439

*No Cq-values detected

*(T)=Triplicates

Discussion

This study had two main aims, first, to investigate if the candidate (miRSeps-7) could be detected and quantified (Absolute quantification) by using the Two-tailed RT-qPCR method developed by Androvic et al (2017). To achieve this, a standard curve (10^6 - 10^{11}) was created from the stock solution (Given by supervisor) of the synthetic candidate miRSeps-7 with copy number 10^{12} . The linear regression equation ($y=mx + b$) retrieved from the SC could be used to calculate the sample concentration. Positive controls (Spiked samples) and negative controls (No-Template-Control, No-Amplification-control, and No-RT) were used to optimize the quality and reproducibility of the experiment (McCall et al., 2014).

The second part of this project was to explore which total RNA extraction method was more efficient between the manual – and the robotic extraction method. This was mainly done by analyzing the Cq-values obtained from the Two-tailed RT-qPCR method. In addition, this was supplemented with A280/A260 absorption ratio (DS-11 spectrophotometer), measurement of the small RNA levels (Qubit), and time management (HOT & TAT) was incorporated into the comparison.

The total RNA extraction

The total RNA extraction may be the most delicate step in the Two-tailed RT-qPCR process since retrieving sufficient amount and quality of circulating miRNA to enable amplification with the

qPCR machine, in this case, miRNA (miRSeqs-7), presents its fair share of challenges (Androvic et al., 2017). The choice of extraction kit is thus a critical step to creating a proper study design.

In a comparative study regarding the extraction of RNA from urinary exosomes, the miRNeasy Serum/Plasma kit (Used in present study) was one of two top performers in terms of efficiency among six prominent RNA extraction kits. In that study, El-Khoury et al (2016) compared the quantity and quality of extracted RNA. All kits produced rather low quantities, however, the miRNeasy Serum/plasma kit, performed particularly well in the quality of the RNA in terms of A260/A280 nm measurement, versus for instance the Trizol® LS. One major reason for this was the phenol residues produced with the Trizol kit which caused contamination. The miRNeasy Serum/Plasma kit does not contain any phenol. (El-Khoury et al., 2016).

QC assessment

The small RNA levels of the samples could not be measured because samples were below the detection limit (0.5ng/ml) of the Qubit® (“Out of range”). This was not a surprise considering miRNA levels are rather low in body fluids (Blondal et al., 2013), in this case, plasma, and previous thesis students' have faced the same challenges, particularly with the low starting material of 100µl of plasma (Aldosaky, 2020; Marinkovic, 2021). Aldosaky (2020) used another extraction kit, but Marinkovic (2021) applied the same kit. Moreover, this predicament could have been managed in the present study, using the LOQ (Limits of quantification) equation (Succop et al., 2004) that has been used previously by students, for instance, Asikainen (2022). However, the LOQ equation was not included in this study. It has been suggested that the risk of incorporating an incorrect LOQ is common, and thus alter the reliability of the test. If not required, theoretical numbers ought to be avoided (Bustin et al., 2009). In a study, conducted by Guo et al (2010), on the prediction intervals for values >LOQ and <LOQ on fat-soluble vitamins. They concluded that the predicted (Theoretical) values, especially for values >LOQ, gave a distorted picture of the true values, and thus were deemed as non-informative. Another solution could be to increase the starting material (Plasma) which was in the present study 100 µl. Nonetheless, a previous publication (Iguchi et al., 2017) used the same extraction kit performed in the present experiment (miRNeasy Serum/Plasma Advanced Handbook). However, they used 200µl, as the protocol suggests, of starting material and they could produce readable results with the Qubit. However, the biological material (plasma) used was not the same as in this experiment; thus, there were different circumstances. The increased starting material does have its obvious advantages with an increased volume of miRNA, however, increased volume, conversely increased volume of inhibitors would alter the quality negatively as well (Chan et al., 2023). Therefore, an optimal starting volume is essential.

Similarly, the use of the A260/A280 ratio, in this case, gives unspecific data since it only measures the total amount of RNA but not the specific miRNA. However, according to the proposed “Minimum Information for Publication of Quantitative Real-Time PCR Experiments” guidelines (MIQE), Qubit’s small RNA quality check and A260/280 (Spectrophotometer) measurements should to be included in the results. Nonetheless, this requirement pertains not when working with very low total RNA values, because they give non-informative information regarding the integrity of the RNA (Bustin et al., 2009). When measuring the A260/280 ratios with microvolume spectrophotometers (MVS), the concentrations of the nucleic acids must be ≥ 20 ng/µl, to get the standard deviation of triplicates, stabilize which is critical for the reproducibility of the conducted testing. Nonetheless, even this number (>20 ng/µl) might be too low and one could argue for a cutoff value of even ≥ 75 ng/µl (Desjardins & Conklin, 2010). Most of the samples in this study, when A260/A280 ratios were measured had a concentration <5.0 ng/µl (Appendix 2, Table 1.), thus not included into the result part of this study. As previously mentioned regarding the “Out of range” measurement for the Qubit, the too-low concentration for was encountered by previous thesis students with the A260/A280 ratio as well (Rozenberg, 2022; Callado Prat, 2023; Norden, 2019; Groenewald, 2022).

However, for the sake of argument, if the concentrations of RNA in this present study were adequate, one may contemplate the unspecific results both Qubit's small RNA assessment gives, and even more so the A260/A280 ratio gathered from the DS-11 spectrophotometer, which indicates the state of the total RNA and not the miRNA. The overall state of the RNA with these two instruments is useful for QC checking if the concentrations are high enough for the nucleic acid. The Qubit is the more precise measurement of the two instruments since it measures small RNA (Wright et al., 2020). However, the advantage the DS-11 spectrophotometer has over Qubit, is that it indicates contaminations within the eluate with the A260/280 ratio (estimation of NA present), and with the A230/260 ratio (estimation of contaminants, e.g. proteins) (Desjardins & Conklin, 2010). The relationship between Cq values and A260/A280 ratios was validated by Ibberson et al (2009), where they analysed how total RNA degradation compromised miR-122 expression profiling with Northern blotting. Moreover, gel-electrophoresis is more informative than DS-11 Spectrophotometer and Qubit when evaluating the overall state and quantity of the RNA. However, the major advantage of using MVS and Qubit over gel-electrophoresis is the simplistic use and quick analysis it provides (Bustin et al., 2009).

As advertised by Androvic et al (2017), including in the present study, the Two-tailed RT-qPCR methods can detect miRNA below the detection limit of Qubit (0.5 ng/ μ l), since otherwise, the miRNA (miRseps-7) would not be converted to cDNA, and consequently the cDNA amplification would have never occurred (Table 5.). Nonetheless, the amplification efficiency of the 20 samples where rather low, since only 10(13) out of 20 samples got amplified. Unfortunately, since the Qubit and DS-11 spectrophotometer could not give any informative details regarding the RNA, this complicates the troubleshooting of this study, on why only 50% (10/20) of samples' cDNA got amplified in the qPCR reactions. The discussion regarding the overall RNA integrity is rather strenuous because, without results from the DS-11 and Qubit, the integrity of which the RNA had at the beginning remains unknown. Consequently, if the low amplification rate was due to compromised RNA, perhaps already from the start (extraction) and/or the quantity/quality diminished during the Two-tailed RT-qPCR. Even though outside the reach of this study, the body fluid from which the RNA is extracted plays a pivotal role in the RNA integrity.

In a study (Max et al., 2018), where miRNA recovery from serum and plasma samples was explored. The researchers collected serum and plasma samples from 13 healthy volunteers, 12 times for two months. They discovered that there was a distinct difference between the overall class of RNA composition. The miRNA, from plasma was captured with a median of 80.9%, compared to serum-derived miRNA with a median of only 54.5% miRNA composition. This means that plasma is the better choice when extracting and analyzing extracellular miRNA for expression profile. Furthermore, the rupture of erythrocytes (Haemolysis), could have a profound effect on the miRNA levels, since a careless extraction of plasma from blood, leads to the release of intracellular miRNA into the extracellular, and thus leads to distorted levels. This was illustrated in one study, conducted by Chan et al (2023), where the evidence showed that the majority of miRNA recovered from plasma was released by haemolysis. Furthermore, haemolysis does not only occur when extracting plasma from blood samples, freezing and thawing processes are believed to induce haemolysis as well. Similarly to Chan et al's conclusion, a review (Bryzgunova, et al 2021), confirmed this notion of the overrepresentation of intracellular miRNA, in body fluids, caused by haemolysis. Additionally, it was also investigated by a previous student (Nordén, 2020), who used the GeneGlobe haemolysis test. The student compared three different RNA extraction kits (Total RNA Purification Kit, miRNeasy Serum/Plasma Kit, and miRNeasy Serum/Plasma Advanced Kit). Nordén (2020) reported that the miRNeasy Serum/Plasma Advanced Kit had a minor advantage, over the other two, in terms of recovery of the RNA isolation.

In this study, RNA degradation could have been a major factor since a dismal quantity of RNA was extracted, too low for Qubit to detect and too low concentrations to give any reliable information

about the state of the total RNA (Malentacchi et al., 2014). RNA is very unstable and degrades very readily, and thus degradation is always part of the equation when extracting RNA from body fluids, in this case plasma (Yamagata et al., 2021). The high quantity of RNAses in human plasma makes the degradation of circular miRNA even more challenging to avoid. Moreover, even though the extraction kit used, did not contain any phenol, the buffers of the extraction kit did contain Guanidine and ethanol, which could be possible contaminants. Additional inhibitory factors of QC check such as DNA and protein may have played an instrumental part as well (Wilfinger et al., 1997).

Another plausible reason for a suboptimal quantity and quality could be the long-term storage of the nucleic acid, which amplifies the deterioration of the miRNA coupled with recurrent freeze - and thawing processes (El-Khoury et al., 2016). In addition, the individual from whom the miRNA has been retrieved from have a massive effect of the amount and sustainability of the nucleic acid. Factors such as diet, exercise, gender and over well-being play an important part. (Becker & Lockwood, 2013). It was also reported in a study (Chomczynski et al., 2016) that the average amount of small RNA in human blood plasma was between 1.91 – 5.29 ng/ μ l. Granted, this study did not use a large sample size (n=35), however, it could perhaps be indicative of the average amount in the population, and thus be an indication of the plasma in the present study that was potentially much-depleted and stored for a prolonged time, and be the reason why small RNA levels were below Qubit's detection limit (0.5ng/ μ l) could not detect.

The pre-analytical factors mentioned above could have had a profound effect on the overall state of the RNA. The non-informative (A260/A280 ratio) and non-generated (Qubit) results could have given more insight into the quality and quantity of the extracted samples. Moreover, with those parameters, it would have been interesting if they corresponded to the cq values generated later on in the pipeline, and if not, perhaps as previously mentioned, possible errors along the way, either at the cDNA synthesis or cDNA amplification could be explanatory.

Hands-On-Time (HOT) and Turn-Around-Time (TAT)

Besides QC check, another parameter to compare the two extraction methods was the estimation of time, since time is of the essence when it comes to sepsis. This was achieved by measuring the HOT and TAT for both methods. To clarify, the measuring of time, or more specifically the TAT, began at the start of the extracting process after the centrifugation at 8000 rpm for 5 minutes. This is because frozen samples are not used within the clinic's diagnostics (Ljungström et al., 2017) and to approximately simulate the real workflow at the hospitals, the timer started at the extraction point. The estimation of time regarding HOT and TAT for the manual and robotic extractions showed no significant differences between the two methods (Table 4.). On average HOT was slightly higher with manual labor (24min) than with the Qiacube (19min), and the TAT, or totality of the whole process, meaning from the start of the extraction to 14 μ l RNA extracts retrieved in a collection tube. On average (Min) the Qiacube (Robotic) took longer (46>42) than the manual extractions. However, since the Qiacube have the capacity of extracting 12 samples simultaneously, these results are not justifiable because only two samples were extracted each time. Consequently, this was the major flaw of this experiment and if done again, should be explored. In other words, the number of samples extracted at the same time could have been investigated, for instance, increasing the sample size by two for each extraction during multiple occasions, going from two to reaching twelve, in turn, would have given an estimation of how much time that could be saved with the machine (Qiacube) with multiple samples, since the hypothesis is that the Qiacube could save time and workload (Sharma et al., 2022). Moreover, the standard deviation (SD) was noticeably greater for the manually extracted samples in both HOT and TAT, and this could be an indication of a more inefficient method, due to the irregular duration of time. In other words, less repeatability. The robotic extraction held a more consistent (Reliable) time, which would give a much easier task to estimate the time for each extraction. Furthermore, the small sample size of total extractions may be indicative of the dismal result as well. In comparison to some previous thesis students, no real trends are observed other than the duration

of robotic extractions is longer on average, how much longer varies. Callado Prat (2023) which used the same extraction kit as present study, with n=54 (n=27 manual, n=27 robotic) total RNA extractions, showed significant differences in the time duration between the robotic (101min) and manual (61min). Furthermore, in one study manual extraction took on average longer (Kasaras, 2022). Nonetheless, the number of samples loaded onto the machine (Qiacube) were not mentioned in these studies, consequently, comparison with these studies does not reveal much, with assumably the same number of extractions performed each time. The point being, logically, if one would increase the number of samples within each extraction, the time would favor the robotic (Qiacube) extraction of each additional sample.

Due to the small sample size in this project (n=12), the same number of samples (n=2) were extracted each time, because of the vastly different results gathered from previous students. Further investigation needs to be done. Again, more data needs to be collected, and as mentioned before, loading the machine and measuring the time, with different numbers of samples.

Two-tailed RT-qPCR

In this study, triplicates and not duplicates were aliquoted onto the qPCR plate, because it is presumably more accurate to correspond to the real variability in technical replicates. A study conducted by Svec et al (2015), reported that using three replicates could improve accuracy by 68%, whereas the use of duplicates improves accuracy by 41%. Consequently, it is highly advantageous to use as many technical replicas as possible. The miRSeqs-7 cDNA did amplify in 10 out of 20 samples, indicating the completion of cDNA synthesis. The ten samples that were amplified underwent a data process where non-informative and off-target samples were filtered out. The non-informative samples that were removed had Cq cutoff values of ≥ 35 and > 0.5 cycles between replicates (de Ronde et al., 2017). Furthermore, to assess the reproducibility of this study, the CV of the samples was calculated (SD/Mean) and none of the duplicates/triplicates had Cq-values $4\% \leq$, which is a marker for solid technical variation (de Ronde et al., 2017). The off-target amplicons could be detected with melt curve analysis and discarded. Amplicons were labeled as off-target when the product displayed melt curve temperature outside of the acceptable range (80-85°C)

Due to the low amount of circulatory miRNA in human plasma, accurate quantification is very challenging. The data is quite difficult to validate and thus hard to compare between different experiments. Researchers are exploring different normalization strategies (Preamplification, RNA isolation etc) but no clear path seems to be agreed upon. The difficulty in comparing miRNA between different samples is the ever-so-challenging reproducibility (Gevaert et al., 2018). As previously mentioned, there is no consensus in the scientific literature regarding how to assess and interpret the qPCR data generated from qPCR with miRNA (Kroh et al., 2010). However, some guidelines seem to be agreed upon which were incorporated into this project (McCall et al., 2014).

Optimal cutoff values are necessary to filter out the non-informative Cq values. The 4RS sample with an Avg Cq >35 (38.3) was discarded and thus deemed as non-informative (e.g. Noise). The off-target amplifications of 4RS are seen in the melt curve graph (Figure 6.). 3MNS and 7RNS were filtered out because of the off-target amplification, seen in the melt curve graphs (Figures 7 & 8). Although one of the replicas of 7RNS had a melt curve of 80.5°C, meaning within range, the majority rule was applied, where 2/3 were not acceptable (McCall et al). The alone acceptable replica had a Cq of 25.89, which could be seen in the raw data collected (Appendix 1, Table 1.). 3MNS had three distinct off-target products, displayed in the melt curve graph (Figure 8.), all three replicas had products with melting temperatures of 87.0 – 87.5°C.

The samples deemed to have no amplification did have amplification in either one replicate or several (Appendix 1. Table 1.). However, the cutoff values failed; the difference between replicates ≥ 0.5 , or/and threshold cycle ≥ 35 . Granted, there are some divergent opinions as it pertains to such

strict cutoff values when dealing with small quantities (de Ronde et al., 2017). The concern is that too much of the data could be filtered out (Removed), and thus detrimental to the statistical power (de Ronde et al., 2017). Oppositely, it has also been suggested that even Cq-values ≥ 30 should be cautiously observed since these high numbers could indicate a non-DNA product (Chan et al., 2023).

Regarding the samples with at least one amplification such as 3RS and 4MS (Appendix 1, Table 1.), one method that could be used is to substitute the missing Cq with one that is equal to the highest possible number of cycles run on the PCR. Nevertheless, this was not implemented in this study because of the extremely low N_0 it generates and thus high likelihood of inappropriate analysis. Similar to the implementation of the LOQ (Limit of equation) equation when dealing with too low concentrations with Qubit, theoretical numbers should always be considered with great caution (McCall et al., 2014).

Primer design is perhaps the most critical step in constructing a proper PCR assay (Robertson & Walsh-Weller, 1998). Unfortunately, the properties of the two-tailed primers used in this project were not disclosed but were informed by the Supervisor at the beginning of the project. The unknown properties include primer sequences, melting temperatures (T_m), and annealing temperature (T_a) (Robertson & Walsh-Weller, 1998). Therefore, the assessment of this project is greatly compromised.

Furthermore, sufficient primers are highly discriminatory between, and in this case toward miRNA isomers, which the two-tailed primers designed by Androvic et al (2017), feature. Sensitivity, or the ability to detect tiny amounts, is equally important as the former (Specificity), especially when working with such small quantities, like miRNA. In addition, temperature tolerance, avoidance of hairpin structures and cross-dimerization are absolute necessities for reliable primers, which ultimately gives higher chances of a reliable PCR assay (Robertson & Walsh-Weller, 1998). As mentioned, no information was disclosed during the project regarding the properties of the primers, thus making a self-assessing discussion about those impossible. However, it was revealed that some previous students had issues with secondary structures, and this was combated by changing the primer concentrations (Personal communication). Secondary structure was indeed experienced in the present project as well, but any experimentation of primer concentration did not take place, due to time restrictions.

At first sight, it appears to be quite feasible to assume that the miRSeps-7 could be detected and quantified (Table 6.), even though with low efficiency, as the SYBR green dye only binds to double-stranded DNA, reinforcing the assumption of the on-target amplification (Arya et al., 2005). Nonetheless, the question is which DNA the two-tailed cDNA primers did bind to, since the SYBR green dye binds unspecific to double-stranded DNA, meaning binds to whichever DNA. According to the melt curve analysis (Figures 6,7 and 8), cDNA miRSeps-7 was not the only product, prompting questions about the efficiency and accuracy of the qPCR. In turn, this relies heavily on the cDNA synthesis as well, which leads back to the design of the target-specific two-tailed RT primers. This is the same case for the target-specific cDNA primers. Nevertheless, what was disclosed was that the manufacturer (Integrated DNA Technologies) had performed proper evaluation and testing on the primers. Moreover, the low amplification rate of the cDNA (7/20) could be a sign of poor primer design (Li & Brownley, 2010). To ensure that the target-specific product, gel electrophoresis or sequence verification are commonly used techniques for conformation of PCR products (Bustin et al., 2017), however, this was not done in this project.

The standard curve had an efficiency of 0.90 and R^2 at 0.99, indicating an acceptable sufficiency (Iguchi, et al 2018) which should have given reliable calculations on the estimated concentrations. However, since several of the samples' estimated Log copy numbers fell outside the range of the standard curve (10^6 >) (Table 6.), the calculations could still be inaccurate, for instance, it is with high possibility that the points outside the range of the SC are nonlinear, meaning that the equation does not apply to them (Androvic et al., 2017). Therefore, one major weakness of the created SC is

the starting point (10^6) of the serial dilution. Instead of $10^6 - 10^{11}$, it would have been more appropriate to have had a SC of $10^2 - 10^8$ or $10^3 - 10^9$. Nonetheless, this was attempted, but not achieved, due to failed attempts and time restrictions.

Absolute quantification was performed on seven spiked samples (1MS, 1RS, 2MS, 2RS, 3MS, 3RS, and 6RS) and on three non-spiked samples (7MNS, 8MNS, and 8RNS). The spiked samples had as expected on average higher quantities of miRSepts-7 due to the addition of $3.5\mu\text{l} \times 10^5$ synthetic miRSepts-7, the log copy numbers ranging from 5.72 to 6.47, and NS-samples from 5.76 to 5.86. (Table 6.). Unfortunately, Cq values from paired samples were not accomplished, as examining the differences between the pair, where the non-spiked samples act as a baseline, and it would have added validity to the quantification (Radonić et al., 2004).

Limitations of the study

Multiple limitations to this study ought to be considered. First, the sample size is far too small to give any substantial statistical power. Perhaps this sample size ($n=20$) would have been enough if they were from one individual. An increase in sample volume would enhance the accuracy and precision of the results (de Ronde et al., 2017). As mentioned above, the lack of paired samples, if done again, it would be beneficial to have spiked- and non-spiked samples performed with both extraction methods from the same plasma sample for all samples. In turn, increases the reproducibility of the experiment (Radonić et al., 2004). Another major limitation of this study was the plethora of unknowns. First, information regarding the candidate miRSepts-7 was not disclosed during the project. What kind of biological processes is it involved with? What biochemical pathways does it regulate? Do the levels of circulatory miRSepts-7 increase or decrease with the contraction of sepsis? etc. Furthermore, the unknown properties and sequences of the designed two-tailed primers (Both RT and cDNA), since the primer sufficiency may be the most critical aspect of the qPCR assay design (Robertson & Walsh-Weller, 1998).

Another limitation of this project was the unknown state of the starting material (Plasma). If the plasma was compromised and the RNA was subjected to high levels of degradation, this could be a plausible explanation for the inefficient qPCR amplification. As previously mentioned, RNA degradation could be induced by several factors such as contaminations, multiple freeze – and thaw processes (El-Khoury et al., 2016). The already natural low amount of miRNA human plasma (Blomdahl et al., 2013), coupled with long-time storage could be a potent combination and reason for so many non-amplifications. The inability to retrieve informative results from the QC check, conducted with the Qubit and DS-11 spectrophotometer, which was experienced by previous students as well (Aldosaky, 2020; Marinkovic, 2021; Callado prat, 2023; Groenewald, 2022, Rozenberg, 2022), is a strong indicator of poor RNA concentration. If the study was done again, the starting volume could be increased to $200\mu\text{l}$, instead of $100\mu\text{l}$ that was used, to compensate for the minuscule RNA levels (Iguchi et al., 2017).

Since no previous student has worked with miRSepts-7, the insufficient amplification could not be compared with another project. The advantages of this would be perhaps to eliminate the notion of that the primers or the biomarker itself could be the problem if another student managed to amplify with greater efficiency than the present study. On the other hand, if the student got similar results, it could be an indication of that the primer design or the biomarker itself was inadequate, this would obviously require that the student used the same biological material, e.g. plasma, as in this study to avoid individual differences in miRNA concentrations.

Ethical aspect and societal impact of this study

This study employs biological materials from consenting non-septic individuals, establishing its ethical foundation. However, caution is warranted in biomarker research to ensure inclusivity across ethnic and gender lines, as miRNA expression has been shown to vary among different ethnicities and genders (Flowers et al., 2022). For instance, a study on miRNA expression and

type-2 diabetes found a strong correlation between high fasting blood glucose (FBG) and miR-126 expression in Hispanic men, which was not observed in at the same rate in other groups (Zampetaki et al., 2010). Notably, the genotypical origin of the plasma used in this study was undisclosed, limiting further commentary. As it pertains to the candidate miRSeps-7, no studies have been conducted on its prevalence in different genotypes, but ought to be in the future. This raises the question of if a multiple biomarker panel could be done for everyone. In the future for group specific or even personalized, may indeed be usable. To tie in with this study, all the non-spiked plasma samples were in close range to each other in terms of copy numbers, perhaps this could be because all the healthy volunteers happened to be from the same group of people (e.g. ethnicity, gender, etc).

This study, which is an incremental part of the "Future Diagnostics of Sepsis" project, holds undisputably profound relevance not only for affected individuals and their families but also for society by large and the environment. Furthermore, increased knowledge of miRNA as a biomarker for sepsis would be also beneficial in the treatment of other illnesses, such as cancer and HIV (Formosa et al., 2022).

Conclusion

The research question of this study was a two-part question; is it more effective to extract RNA manually or robotically? Effectiveness is defined as how fast (in minutes) the process is, and more importantly with what degree of accuracy (Cq-value) it can be performed. The experimental findings in this project failed to answer this question. In large part because of the small sample size, in combination with poor execution, where Qiacube's load (n=12) capacity was not taken into consideration.

The second part of the two-part question; is the candidate detectable and quantifiable when using the two-tailed RT-qPCR technique with the implementation of the absolute quantification method? The answer to this question is somewhat more complicated than the answer to the first question. Amplification (detection) was achieved on 7/20 samples with the Two-tailed RT-qPCR. However, what was amplified? Since this was not confirmed with instruments such as gel electrophoresis or sequence verification (Bustin et al., 2017). Without verification, it is impossible to be 100% certain that the miRSeps-7 was product that was detected and quantified in the human plasma.

Acknowledgement

I would like to give a special thanks to my supervisor Anna-Karin Pernestig and my co-supervisor Nada Mahmoud for their guidance and feedback given throughout this project. I would also like to acknowledge my co-workers, who helped to shape an enjoyable environment to be in both in- and outside the laboratory.

References

- Androvic, P., Valihrach, L., Elling, J., Sjoback, R., & Kubista, M. (2017). Two-tailed RT-qPCR: a novel method for highly accurate miRNA quantification. *Nucleic acids research*, *45*(15), e144. <https://doi.org/10.1093/nar/gkx588>
- Antonakos, N., Gilbert, C., Théroude, C., Schrijver, I. T., & Roger, T. (2022). Modes of action and diagnostic value of miRNAs in sepsis. *Frontiers in immunology*, *13*, 951798. <https://doi.org/10.3389/fimmu.2022.951798>
- Arya, M., Shergill, I. S., Williamson, M., Gommersall, L., Arya, N., & Patel, H. R. (2005). Basic principles of real-time quantitative PCR. *Expert review of molecular diagnostics*, *5*(2), 209–219. <https://doi.org/10.1586/14737159.5.2.209>
- Becker, N., & Lockwood, C. M. (2013). Pre-analytical variables in miRNA analysis. *Clinical biochemistry*, *46*(10-11), 861–868. <https://doi.org/10.1016/j.clinbiochem.2013.02.015>
- Brunet-Vega, A., Pericay, C., Quílez, M. E., Ramírez-Lázaro, M. J., Calvet, X., & Lario, S. (2015). Variability in microRNA recovery from plasma: Comparison of five commercial kits. *Analytical biochemistry*, *488*, 28–35. <https://doi.org/10.1016/j.ab.2015.07.018>
- Bryzgunova, O., Konoshenko, M., Zaporozhchenko, I., Yakovlev, A., & Laktionov, P. (2021). Isolation of Cell-Free miRNA from Biological Fluids: Influencing Factors and Methods. *Diagnostics (Basel, Switzerland)*, *11*(5), 865. <https://doi.org/10.3390/diagnostics11050865>
- Centers for Medicare & Medicaid Services (5 September 2023). Medicare inpatient hospitals. <https://data.cms.gov/provider-summary-by-type-of-service/medicare-inpatient-hospitals>
- Chan, S. F., Cheng, H., Goh, K. K., & Zou, R. (2023). Preanalytic Methodological Considerations and Sample Quality Control of Circulating miRNAs. *The Journal of molecular diagnostics : JMD*, *25*(7), 438–453. <https://doi.org/10.1016/j.jmoldx.2023.03.005>
- Chomczynski, P., Wilfinger, W. W., Eghbalnia, H. R., Kennedy, A., Rymaszewski, M., & Mackey, K. (2016). Inter-Individual Differences in RNA Levels in Human Peripheral Blood. *PLoS one*, *11*(2), e0148260. <https://doi.org/10.1371/journal.pone.0148260>
- Desjardins, P., & Conklin, D. (2010). NanoDrop microvolume quantitation of nucleic acids. *Journal of visualized experiments : JoVE*, (45), 2565. <https://doi.org/10.3791/2565>
- El-Khoury, V., Pierson, S., Kaoma, T., Bernardin, F., & Berchem, G. (2016). Assessing cellular and circulating miRNA recovery: the impact of the RNA isolation method and the quantity of input material. *Scientific reports*, *6*, 19529. <https://doi.org/10.1038/srep19529>
- de Ronde, M. W. J., Ruijter, J. M., Lanfear, D., Bayes-Genis, A., Kok, M. G. M., Creemers, E. E., Pinto, Y. M., & Pinto-Sietsma, S. J. (2017). Practical data handling pipeline improves performance of qPCR-based circulating miRNA measurements. *RNA (New York, N.Y.)*, *23*(5), 811–821. <https://doi.org/10.1261/rna.059063.116>
- Evans, L., Rhodes, A., Alhazzani, W., Antonelli, M., Coopersmith, C. M., French, C., Machado, F. R., McIntyre, L., Ostermann, M., Prescott, H. C., Schorr, C., Simpson, S., Wiersinga, W. J., Alshamsi, F., Angus, D. C., Arabi, Y., Azevedo, L., Beale, R., Beilman, G., Belley-Cote, E., ... Levy, M. (2021). Surviving sepsis campaign: international guidelines for management of sepsis and septic shock 2021. *Intensive care medicine*, *47*(11), 1181–1247. <https://doi.org/10.1007/s00134-021-06506-y>
- Fauth, M., Hegewald, A. B., Schmitz, L., Krone, D. J., & Saul, M. J. (2019). Validation of extracellular miRNA quantification in blood samples using RT-qPCR. *FASEB bioAdvances*, *1*(8), 481–492. <https://doi.org/10.1096/fba.2019-00018>

- Fleige, S., Walf, V., Huch, S., Prgomet, C., Sehm, J., & Pfaffl, M. W. (2006). Comparison of relative mRNA quantification models and the impact of RNA integrity in quantitative real-time RT-PCR. *Biotechnology letters*, 28(19), 1601–1613. <https://doi.org/10.1007/s10529-006-9127-2>
- Flowers, E., Kanaya, A. M., Zhang, L., & Aouizerat, B. E. (2022). The Role of Racial and Ethnic Factors in MicroRNA Expression and Risk for Type 2 Diabetes. *Frontiers in genetics*, 13, 853633. <https://doi.org/10.3389/fgene.2022.853633>
- Formosa, A., Turgeon, P., & Dos Santos, C. C. (2022). Role of miRNA dysregulation in sepsis. *Molecular medicine (Cambridge, Mass.)*, 28(1), 99. <https://doi.org/10.1186/s10020-022-00527-z>
- Gagnon, K. T., Li, L., Chu, Y., Janowski, B. A., & Corey, D. R. (2014). RNAi factors are present and active in human cell nuclei. *Cell reports*, 6(1), 211–221. <https://doi.org/10.1016/j.celrep.2013.12.013>
- Garcia-Elias, A., Alloza, L., Puigdecenet, E. et al. Defining quantification methods and optimizing protocols for microarray hybridization of circulating microRNAs. *Sci Rep* 7, 7725 (2017). <https://doi.org/10.1038/s41598-017-08134-3>
- Ghasemi, A., & Zahediasl, S. (2012). Normality tests for statistical analysis: a guide for non-statisticians. *International journal of endocrinology and metabolism*, 10(2), 486–489. <https://doi.org/10.5812/ijem.3505>
- Git, A., Dvinge, H., Salmon-Divon, M., Osborne, M., Kutter, C., Hadfield, J., Bertone, P., & Caldas, C. (2010). Systematic comparison of microarray profiling, real-time PCR, and next-generation sequencing technologies for measuring differential microRNA expression. *RNA (New York, N.Y.)*, 16(5), 991–1006. <https://doi.org/10.1261/rna.1947110>
- (Guo, Y., Harel, O., & Little, R. J. (201. How well quantified is the limit of quantification?. *Epidemiology (Cambridge, Mass.)*, 21 Suppl 4, S10–S16. <https://doi.org/10.1097/EDE.0b013e3181d60e56>)
- Ho, P. T. B., Clark, I. M., & Le, L. T. T. (2022). MicroRNA-Based Diagnosis and Therapy. *International journal of molecular sciences*, 23(13), 7167. <https://doi.org/10.3390/ijms23137167>
- Ibberson, D., Benes, V., Muckenthaler, M. U., & Castoldi, M. (2009). RNA degradation compromises the reliability of microRNA expression profiling. *BMC biotechnology*, 9, 102. <https://doi.org/10.1186/1472-6750-9-102>
- Iguchi, T., Niino, N., Tamai, S., Sakurai, K., & Mori, K. (2018). Absolute Quantification of Plasma MicroRNA Levels in Cynomolgus Monkeys, Using Quantitative Real-time Reverse Transcription PCR. *Journal of visualized experiments : JoVE*, (132), 56850. <https://doi.org/10.3791/56850>
- Iwashyna, T. J., Ely, E. W., Smith, D. M., & Langa, K. M. (2010). Long-term cognitive impairment and functional disability among survivors of severe sepsis. *JAMA*, 304(16), 1787–1794. <https://doi.org/10.1001/jama.2010.1553>
- Krepelkova, I., Mrackova, T., Izakova, J., Dvorakova, B., Chalupova, L., Mikulik, R., Slaby, O., Bartos, M., & Ruzicka, V. (2019). Evaluation of miRNA detection methods for the analytical characteristic necessary for clinical utilization. *BioTechniques*, 66(6), 277–284. <https://doi.org/10.2144/btn-2019-0021>
- Kroh, E. M., Parkin, R. K., Mitchell, P. S., & Tewari, M. (2010). Analysis of circulating microRNA biomarkers in plasma and serum using quantitative reverse transcription-PCR (qRT-PCR). *Methods (San Diego, Calif.)*, 50(4), 298–301. <https://doi.org/10.1016/j.ymeth.2010.01.032>
- Kumar, A., Roberts, D., Wood, K. E., Light, B., Parrillo, J. E., Sharma, S., Suppes, R., Feinstein, D., Zanotti, S., Taiberg, L., Gurka, D., Kumar, A., & Cheang, M. (2006). Duration of hypotension before

initiation of effective antimicrobial therapy is the critical determinant of survival in human septic shock. *Critical care medicine*, 34(6), 1589–1596. <https://doi.org/10.1097/01.CCM.0000217961.75225.E9>

Lee, R. C., Feinbaum, R. L., & Ambros, V. (1993). The *C. elegans* heterochronic gene *lin-4* encodes small RNAs with antisense complementarity to *lin-14*. *Cell*, 75(5), 843–854. [https://doi.org/10.1016/0092-8674\(93\)90529-y](https://doi.org/10.1016/0092-8674(93)90529-y)

Liu, C. F., Shi, X. P., Chen, Y., Jin, Y., & Zhang, B. (2018). Rapid diagnosis of sepsis with TaqMan-Based multiplex real-time PCR. *Journal of clinical laboratory analysis*, 32(2), e22256. <https://doi.org/10.1002/jcla.22256>

Ljungström, L., Andersson, R., & Jacobsson, G. (2019). Incidences of community onset severe sepsis, Sepsis-3 sepsis, and bacteremia in Sweden - A prospective population-based study. *PloS one*, 14(12), e0225700. <https://doi.org/10.1371/journal.pone.0225700>

Malentacchi, F., Pazzagli, M., Simi, L., Orlando, C., Wyrich, R., Günther, K., Verderio, P., Pizzamiglio, S., Ciniselli, C. M., Zhang, H., Korenková, V., Rainen, L., Bar, T., Kubista, M., & Gelmini, S. (2014). SPIDIA-RNA: second external quality assessment for the pre-analytical phase of blood samples used for RNA based analyses. *PloS one*, 9(11), e112293. <https://doi.org/10.1371/journal.pone.0112293>

Max, K. E. A., Bertram, K., Akat, K. M., Bogardus, K. A., Li, J., Morozov, P., Ben-Dov, I. Z., Li, X., Weiss, Z. R., Azizian, A., Sopeyin, A., Diacovo, T. G., Adamidi, C., Williams, Z., & Tuschl, T. (2018). Human plasma and serum extracellular small RNA reference profiles and their clinical utility. *Proceedings of the National Academy of Sciences of the United States of America*, 115(23), E5334–E5343. <https://doi.org/10.1073/pnas.1714397115>

McCall, M. N., McMurray, H. R., Land, H., & Almudevar, A. (2014). On non-detects in qPCR data. *Bioinformatics (Oxford, England)*, 30(16), 2310–2316. <https://doi.org/10.1093/bioinformatics/btu239>

McDonald, J. S., Milosevic, D., Reddi, H. V., Grebe, S. K., & Algeciras-Schimnich, A. (2011). Analysis of circulating microRNA: preanalytical and analytical challenges. *Clinical chemistry*, 57(6), 833–840. <https://doi.org/10.1373/clinchem.2010.157198>

Mestdagh, P., Feys, T., Bernard, N., Guenther, S., Chen, C., Speleman, F., & Vandesompele, J. (2008). High-throughput stem-loop RT-qPCR miRNA expression profiling using minute amounts of input RNA. *Nucleic acids research*, 36(21), e143. <https://doi.org/10.1093/nar/gkn725>

Mishra, P., Pandey, C. M., Singh, U., Gupta, A., Sahu, C., & Keshri, A. (2019). Descriptive statistics and normality tests for statistical data. *Annals of cardiac anaesthesia*, 22(1), 67–72. https://doi.org/10.4103/aca.ACA_157_18

Nettle, D., Seeker, L., Nussey, D., Froy, H., & Bateson, M. (2019). Consequences of measurement error in qPCR telomere data: A simulation study. *PloS one*, 14(5), e0216118. <https://doi.org/10.1371/journal.pone.0216118>

Prescott, H. C., & Angus, D. C. (2018). Enhancing Recovery From Sepsis: A Review. *JAMA*, 319(1), 62–75. <https://doi.org/10.1001/jama.2017.17687>

Radonić, A., Thulke, S., Mackay, I. M., Landt, O., Siegert, W., & Nitsche, A. (2004). Guideline to reference gene selection for quantitative real-time PCR. *Biochemical and biophysical research communications*, 313(4), 856–862. <https://doi.org/10.1016/j.bbrc.2003.11.177>

Robertson, J.M., Walsh-Weller, J. (1998). An Introduction to PCR Primer Design and Optimization of Amplification Reactions. In: Lincoln, P.J., Thomson, J. (eds) *Forensic DNA Profiling Protocols. Methods in Molecular Biology*, vol 98. Humana Press. <https://doi.org/10.1385/0-89603-443-7:121>

- Rogobete, A. F., Sandesc, D., Bedreag, O. H., Papurica, M., Popovici, S. E., Bratu, T., Popoiu, C. M., Nitu, R., Dragomir, T., AAbed, H. I. M., & Ivan, M. V. (2018). MicroRNA Expression is Associated with Sepsis Disorders in Critically Ill Polytrauma Patients. *Cells*, 7(12), 271. <https://doi.org/10.3390/cells7120271>
- Seelenfreund, E., Robinson, W. A., Amato, C. M., Tan, A. C., Kim, J., & Robinson, S. E. (2014). Long term storage of dry versus frozen RNA for next generation molecular studies. *PloS one*, 9(11), e111827. <https://doi.org/10.1371/journal.pone.0111827>
- Sharma, P., Singh, M., Singh, A., Bhardwaj, D., & Bhatia, P. (2022). Experience of quantity and quality of DNA and RNA extraction from limited pediatric blood samples: A comparative analysis of automated and manual kit-based method. *Indian journal of pathology & microbiology*, 65(1), 105–110. https://doi.org/10.4103/IJPM.IJPM_946_20
- Singer, M., Deutschman, C. S., Seymour, C. W., Shankar-Hari, M., Annane, D., Bauer, M., Bellomo, R., Bernard, G. R., Chiche, J. D., Cooper-Smith, C. M., Hotchkiss, R. S., Levy, M. M., Marshall, J. C., Martin, G. S., Opal, S. M., Rubenfeld, G. D., van der Poll, T., Vincent, J. L., & Angus, D. C. (2016). The Third International Consensus Definitions for Sepsis and Septic Shock (Sepsis-3). *JAMA*, 315(8), 801–810. <https://doi.org/10.1001/jama.2016.0287>
- Succop, P. A., Clark, S., Chen, M., & Galke, W. (2004). Imputation of data values that are less than a detection limit. *Journal of occupational and environmental hygiene*, 1(7), 436–441. <https://doi.org/10.1080/15459620490462797>
- Svec, D., Tichopad, A., Novosadova, V., Pfaffl, M. W., & Kubista, M. (2015). How good is a PCR efficiency estimate: Recommendations for precise and robust qPCR efficiency assessments. *Biomolecular detection and quantification*, 3, 9–16. <https://doi.org/10.1016/j.bdq.2015.01.005>
- Tili, E., Michaille, J. J., Cimino, A., Costinean, S., Dumitru, C. D., Adair, B., Fabbri, M., Alder, H., Liu, C. G., Calin, G. A., & Croce, C. M. (2007). Modulation of miR-155 and miR-125b levels following lipopolysaccharide/TNF-alpha stimulation and their possible roles in regulating the response to endotoxin shock. *Journal of immunology (Baltimore, Md. : 1950)*, 179(8), 5082–5089. <https://doi.org/10.4049/jimmunol.179.8.5082>
- Torio CM, Andrews RM. National Inpatient Hospital Costs: The Most Expensive Conditions by Payer, 2011. 2013 Aug. In: Healthcare Cost and Utilization Project (HCUP) Statistical Briefs [Internet]. Rockville (MD): Agency for Healthcare Research and Quality (US); 2006 Feb-. Statistical Brief #160. Available from: <https://www.ncbi.nlm.nih.gov/books/NBK169005/>
- Vasilescu, C., Rossi, S., Shimizu, M., Tudor, S., Veronese, A., Ferracin, M., Nicoloso, M. S., Barbarotto, E., Popa, M., Stanciulea, O., Fernandez, M. H., Tulbure, D., Bueso-Ramos, C. E., Negrini, M., & Calin, G. A. (2009). MicroRNA fingerprints identify miR-150 as a plasma prognostic marker in patients with sepsis. *PloS one*, 4(10), e7405. <https://doi.org/10.1371/journal.pone.0007405>
- 1177 Vårdguiden.(10 February 2023). Sepsis – blodförgiftning. <https://www.1177.se/sjukdomar-besvar/infektioner/feber/sepsis---blodforgiftning/>
- Want, A., Staniak, K., Grabowska-Pyrzewicz, W., Fesiuk, A., Barczak, A., Gabryelewicz, T., Kulczyńska-Przybik, A., Mroczko, B., & Wojda, U. (2023). Optimized RT-qPCR and a novel normalization method for validating circulating miRNA biomarkers in ageing-related diseases. *Scientific reports*, 13(1), 20869. <https://doi.org/10.1038/s41598-023-47971-3>
- Willenbrock, H., Salomon, J., Søkilde, R., Barken, K. B., Hansen, T. N., Nielsen, F. C., Møller, S., & Litman, T. (2009). Quantitative miRNA expression analysis: comparing microarrays with next-generation sequencing. *RNA (New York, N.Y.)*, 15(11), 2028–2034. <https://doi.org/10.1261/rna.1699809>

- Wilfinger, W. W., Mackey, K., & Chomczynski, P. (1997). Effect of pH and Ionic Strength on the Spectrophotometric Assessment of Nucleic Acid Purity. *BioTechniques*, 22(3), 474–481. <https://doi.org/10.2144/97223st01>
- Wright, K., de Silva, K., Purdie, A. C., & Plain, K. M. (2020). Comparison of methods for miRNA isolation and quantification from ovine plasma. *Scientific reports*, 10(1), 825. <https://doi.org/10.1038/s41598-020-57659-7>
- Yamagata, H., Kobayashi, A., Tsunedomi, R., Seki, T., Kobayashi, M., Hagiwara, K., Chen, C., Uchida, S., Okada, G., Fuchikami, M., Kamishikiryo, T., Iga, J. I., Numata, S., Kinoshita, M., Kato, T. A., Hashimoto, R., Nagano, H., Okamoto, Y., Ueno, S., Ohmori, T., ... Nakagawa, S. (2021). Optimized protocol for the extraction of RNA and DNA from frozen whole blood sample stored in a single EDTA tube. *Scientific reports*, 11(1), 17075. <https://doi.org/10.1038/s41598-021-96567-2>
- Zampetaki, A., Kiechl, S., Drozdov, I., Willeit, P., Mayr, U., Prokopi, M., Mayr, A., Weger, S., Oberhollenzer, F., Bonora, E., Shah, A., Willeit, J., & Mayr, M. (2010). Plasma microRNA profiling reveals loss of endothelial miR-126 and other microRNAs in type 2 diabetes. *Circulation research*, 107(6), 810–817. <https://doi.org/10.1161/CIRCRESAHA.110.226357>
- Zampetaki, A., & Mayr, M. (2012). Analytical challenges and technical limitations in assessing circulating miRNAs. *Thrombosis and haemostasis*, 108(4), 592–598. <https://doi.org/10.1160/TH12-02-0097>
- Zhang, J., Li, S., Li, L., Li, M., Guo, C., Yao, J., & Mi, S. (2015). Exosome and exosomal microRNA: trafficking, sorting, and function. *Genomics, proteomics & bioinformatics*, 13(1), 17–24. <https://doi.org/10.1016/j.gpb.2015.02.001>

Appendix 1

Table 1. Raw data of Cq-values retrieved from qPCR reaction of each sample in triplicates.

Samples	1	2	3
1MS	24.14	24.80	24.19
1RS	27.82	25.08	25.58
2MS	24.61	24.46	No Cq
2RS	23.82	No Cq	24.04
3MS	No Cq	30.80	No Cq
3RS	37.90	No Cq	No Cq
4MS	34.93	No Cq	No Cq
4RS	38.30	38.33	35.36
5MS	26.75	26.61	26.42
3MNS	32.89	32.72	32.52
5RS	25.58	24.68	25.12
6MS	27.05	31.12	26.01
6RS	26.85	26.77	26.77
7MNS	26.07	26.07	31.69
7RNS	31.89	31.51	25.89
8MNS	26.64	26.22	26.38
8RNS	31.68	26.60	26.26

Appendix 2

Table 1. Measurement of all samples (n=20) retrieved from the DeNovix DS-11 Spectrophotometer

Sample	ng/μl	A260	A260/A230	A260/A280
1MS	0.835	0.021	0.172	0.720
1RS	11.911	0.298	0.607	1.582
2MS	1.899	0.048	0.139	1.153
2RS	2.066	0.052	0.814	1.516
3MS	0.073	0.018	0.059	0.799
3RS	19.571	0.489	0.428	1.523
4MS	2.070	0.0518	0.026	2.594
4RS	0.211	0.005	0.010	0.386
5MS	2.684	0.067	0.012	1.277
5RS	0.097	0.024	0.013	1.318
6MS	3.795	0.095	0.016	1.643
6RS	1.155	0.0289	0.153	3.258
7MNS	0.404	0.010	0.009	0.0539
7RNS	1.032	0.026	0.283	1.827
8MNS	2.632	0.0658	0.017	2.037
8RNS	1.548	0.085	0.673	1.218