Degree project

UNIVERSITY OF SKÖVDE

Evaluation of the Two-tailed RT-qPCR method with the application of manual vs robotic extraction of miRNA

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Student: Lia Rozenberg
a19polro@student.his.se

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

Examiner: Magnus Fagerlind
magnus.fagerlind@his.se

Abstract

Sepsis is aggressive and severe inflammatory body response to an infection and is considered to be one of the most common death causes in patients. The current diagnosis of sepsis is not fast enough to help those who get sepsis, due to its fast progression. The current golden standard for sepsis diagnosis is blood culturing. However, the biggest downside of it is the long time. Research is now focused on finding a faster way to diagnose sepsis on early stages. The most promising one tends to be the usage of biomarkers. Today, there are 260 defined sepsis biomarkers, however, only few of them are clinically used. Among them, C-reactive protein, and procalcitonin. Another potential biomarker could be miRNAs. The research about that today is at early stage. To use miRNAs as biomarkers, they need to be quantified. One way to quantify miRNAs is the two-tailed RT-qPCR method together with absolute quantification. This study focused on evaluating the best extraction method of small RNA for later quantification of specific miRNA. The blood plasma from healthy donors was divided into spiked and non-spiked samples, where the synthetic miRSeps-3 served as a spike-in positive controls. All samples were extracted using two methods, manual and robotic with Qiacube (Qiagen). Absolute quantification was applied to quantify miRNA in all samples. The successful results indicated that the two-tailed RT-qPCR was sensitive enough. More optimization is required for the methods, however, the whole method has a good potential to become helpful for clinical usage in the future.

List of abbreviations

cDNA	Complementary deoxyribonucleic acid
CRP	C-reactive protein
Cq	Quantitative cycle
DNA	Deoxyribonucleic acid
ELISA	Enzyme-linked immunosorbent assay
ICU	Intensive Unit Care
IPC	Inter-plate calibrator
LOQ	Limit of quantification
miRNA	Micro ribonucleic acid
NGS	Next Generation Sequencing
NTC	No template control
PCR	Polymerase chain reaction
PCT	Procalcitonin
QC	Quality control
qPCR	Quantitative polymerase chain reaction
RNA	Ribonucleic acid
RT	Reverse transcription
-RT	No reverse transcriptase
RT-qPCR	Quantitative reverse transcription polymerase chain reaction
R^2	Coefficient of determination
SD	Standard deviation
SIRS	Systemic inflammatory response syndrome
SOFA	Sequential organ failure assessment

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Introduction

Sepsis

Sepsis is a very aggressive and severe inflammatory body response to an infection and is considered to be one of the most common death causes in patients (Salomão et al., 2019). Sepsis is a worldwide major medical issue, even in developed countries. The number of patients, especially in the intensive care unit (ICU) worldwide, getting sepsis with a fatal ending is around 26% (Danai et al, 2005; Sakr et al, 2018). The definition of sepsis has changed throughout the years, due to it being hard to define. Before 2016, sepsis was defined as a condition that usually evolves rapidly, first causing a systemic inflammatory response syndrome (SIRS), followed by sepsis which causes multiorgan dysfunction and eventually septic shock. A septic shock condition is when blood pressure is extremely low, approximately 90/60 mmHg and below being one of the parameters, which results in death (Essandoh and Fan, 2014; Marik and Lipman, 2007). In 2016, the newest definition of sepsis was established, which is called Sepsis-3 (Gul et al., 2017). Sepsis-3 is now defined as life-threatening organ dysfunction caused by a dysregulated host response to infection. The main reason behind changing the definition was because of symptoms of SIRS are not specific and sensitive enough and can change in patients depending on many factors (Gul et al., 2017). In order to be defined as a septic patient, a sequential organ failure assessment (SOFA) scoring should be followed (Marik and Taeb, 2017). The SOFA follows scores in six categories, namely, respiratory, cardiovascular, hepatic, coagulation, renal, and neurological systems, from 0 to 4, where 4 is the highest and signals about major organ disfunction (Lambden, Laterre, Levy, and Francois, 2019). SOFA score considered to be simple but effective method to divide patients in ICUs into risk groups for further therapy (Jain et al., 2016).

Diagnosis of sepsis

The current diagnosis of sepsis is not nearly fast enough to help those who get sepsis, due to its fast progression. Besides the obvious signs of SIRS, such as fever (>38°C or <36°C), tachycardia (>90 heartbeats per minute), tachypnea (>20 breaths per minute), and abnormal white blood cell count, that can be detected in patients and an immediate antibiotic treatment that is given to them (Husabø et al., 2021; Comstedt et al., 2009), the most common sepsis diagnosis way is the blood culturing. The downside of this method is the long waiting time, which takes up to 48 hours. Besides, the quantity of bacterial colonies being low as well as the insensitivity of the cultures (Opota et al., 2015), which in general results in time being the most important criteria that need to be changed to be able to diagnose sepsis earlier. Among blood culturing, there are other approaches in the early diagnosis of sepsis, such as performing PCR tests, ELISA kits, flow cytometry and other techniques (Chan et al., 2011). The most promising one tends to be the biomarker approach, due to the ability to monitor the abnormal changes in that specific biomarker (Chan et al., 2011). There is a large amount of known sepsis biomarkers, 260 identified, to be exact (Pierrakos et al., 2020) but very few are currently used in clinical laboratories, among them, C-reactive protein (CRP), proinflammatory cytokines (such as IL-1 and IL-6) and procalcitonin (PCT) being the most common ones (Fabri-Faja et al., 2019).

miRNAs as a biomarkers

The use of microRNA (miRNA) as potential biomarkers for sepsis diagnosis is the study of interest nowadays, due to the miRNAs being non-protein-coding RNAs (~22 nt) that regulate the expression of target mRNA after transcription as well as for their diagnostic potential in other diseases and infections. The miRNAs are related to several processes in the body and their expression can be served as a diagnostic biomarker for the early diagnosis of illnesses (Wang et al., 2013). The miRNAs are usually available in large quantities for tissues but are in limited amounts (6-300 ng/ml) in serum and plasma (Jensen et al., 2011). In a study, that Wang et al

(2013) were performing, the level of a certain miRNA, more specifically, miR-146a, was lower in serum/plasma when it came to septic patients, in comparison to healthy individuals. According to Essandoh and Fan (2014), there have been many studies about the idea of using extracellular miRNAs as biomarkers, however, only miR-223, miR-146, and miR-150 are promising in the prognosis and diagnosis of sepsis.

Qiacube

The extraction of miRNA from fluids, such as plasma may be problematic due to the low amount and their short size (Moret et al., 2013). There are several available kits for miRNA extraction from different body fluids, such as plasma, serum, and studies have been made in order to compare the kits for. For example, in a study that Moret et al (2013) did, the most efficient kit, that gave the best result in both quantity and quality for further analysis of the extracted miRNA, was the miRNeasy serum/plasma kit from Qiagen. Since the miRNA is a promising candidate as a diagnostic marker, the clinical labs demand an extraction method that can be fast and secure. One possibility for extraction is the usage of the Qiacube from Qiagen. Qiacube is a robotic system that can purify nucleic acid and proteins (McGraw et al., 2014). Qiagen offers 140 standard protocols that can be used with Qiacube, but also, there is a possibility for protocol customization, which means, the machine can be programmed to specific needs (Qiagen, 2022). In general, reducing hands-on time and getting the very same quality control results every time are the main goals of robotic extraction. It is important to optimize the robotic extraction method to land in the same or higher yield of quality and quantity of the product, as in the manual extraction method, and to reduce hands-on time spent on it for clinical laboratory workers.

Two-tailed RT-qPCR

In order to quantify the extracted miRNA, the most common methods in use, according to Androvic et al (2017), are microarrays, Next Generation Sequencing (NGS), and reverse transcription-quantitative polymerase chain reaction (RT-qPCR). RT-qPCR is considered one of the best (Pombo et al., 2017) methods at the moment, due to its sensitivity, accuracy and rapid execution, to validate the miRNA expression profiling, but it becomes a high-cost method when it comes to measurement of large number of miRNA targets. Androvic et al (2017) have developed a cost-friendly and highly effective method called two-tailed RT-qPCR (Figure 1). The idea behind it is that the new design of RT primers should allow them to hybridize at two regions of the miRNA having two separate complementary parts (hemiprobes). One of the advantages of the two-tailed RT primer is that the 3´-hemiprobe can be designed to be short, which eventually will allow the making of a miRNA-specific qPCR primer, due to having free space and not overlapping with the 3´-hemiprobe.

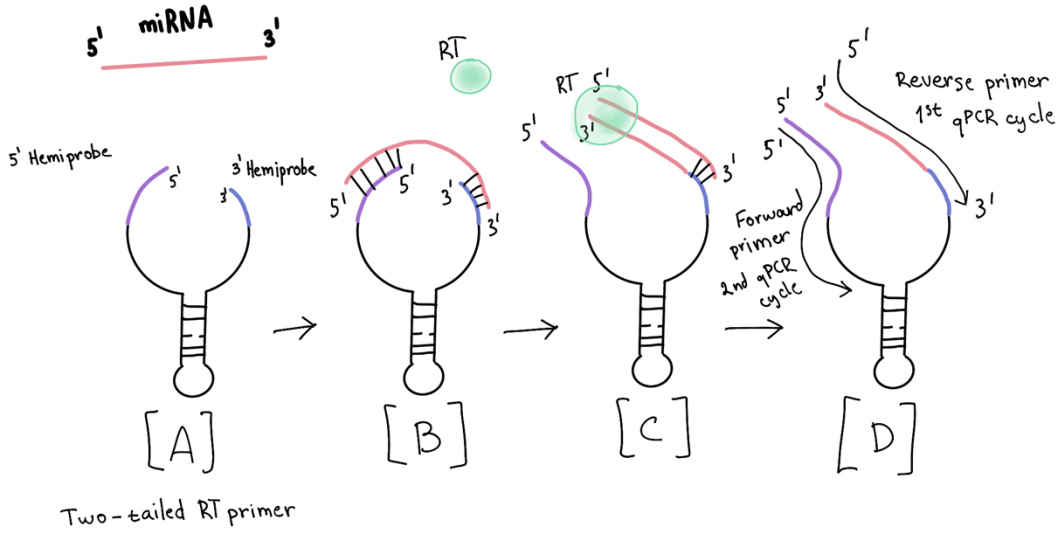

Figure 1. The figure shows the two-tailed RT primer that in [A] the primer has two hemiprobes which are connected with a hairpin sequence, [B] the miRNA binds to the end accordingly, [C] shows the RT enzyme binding to the 3'-end, and extend it with RT, [D] shows the cDNA amplification with the target-specific primers.

Aim

The aim of this project was to compare two extraction methods, namely, robotic with help of automated pipetting machine Qiacube and manual extraction completely made by hand. For this, the miRNeasy Serum/Plasma Advanced Kit (Qiagen) was used for both methods to be able to compare the results in quality and quantity of extracted small RNAs. The second part of the project aimed to quantify the extracted miRNA with help of Two-tailed RT-qPCR methods together with absolute quantification, as well as see whether the method itself would be able to detect the extracted miRNA.
The current stage of research about miRNA as a potential biomarker is still under development. This project is a part of the bigger ongoing research within sepsis field, called "Future diagnostic of sepsis". The research goal is to create a multimarker panel that would ease the diagnosis of sepsis on early stages. It is of interest to find more potential biomarkers for the multimarker panel, hence this thesis project assisted with it by trying out the methods on a synthetic miRNA.

How are concentration and purity of the miRNA affected and how do they differentiate when the miRNAs are extracted fully automated by Qiacube vs handling the extraction by hand?

Is it possible for the Two-tailed RT-qPCR method to detect the miRNA in plasma?

Objectives of this study:

- Extract plasma from healthy blood
- Extract miRNA from plasma both manually and robotically

- Perform quality control (QC)
- Perform the Two-tailed RT-qPCR method and absolute quantification
- Statistically analyse the QC results

Material & Methods

The workflow of the project

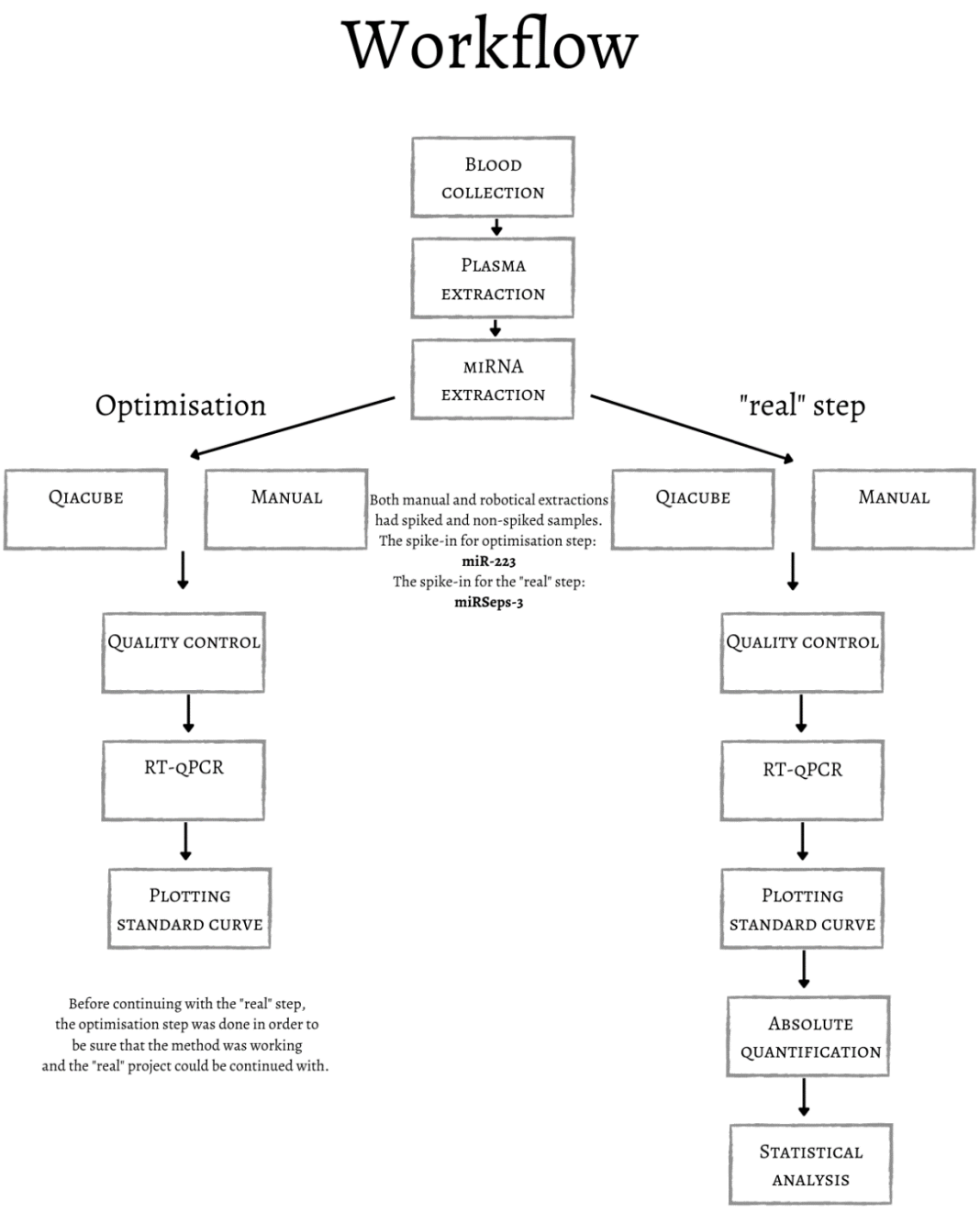

Figure 2. The overview of the workflow used through the whole project. The left side shows the optimization flow, while the right side shows the flow followed after the optimization step.

Blood collection and plasma extraction

Whole blood was collected from healthy human volunteers in prepared and labelled 4 ml Vacuette K2E EDTA tubes (Greiner Bio-One). The EDTA tubes were inverted ten times and stored on ice

upon centrifugation. Later, the tubes were centrifuged at 1790 rcf for 15 minutes, according to ThermoFisher Scientific in ScanSpeed 1580R centrifuge (Labogene). The separated plasma was later collected and divided into 1.5 ml Eppendorf tubes with each tube containing 500 µl of plasma and stored at -80°C.

Total RNA extraction

RNA extraction was performed where both spiked-in, with synthetic miRSeps-3 (Integrated DNA technologies) in concentrations 10^4, 10^5, and 10^6 copies/µl per four samples, as well as non-spiked extractions were executed. For the optimization step, the synthetic miR-223 was used. Extractions were executed in two different ways, manual and robotic. The manual procedure followed the miRNeasy Serum/Plasma Advanced handbook protocol (Qiagen) with a change at the beginning in the amount of added plasma, which was 100 µl instead of 200 µl. Due to the changed amount of plasma, the amount of RPL and RPP buffers were changed from 60 µl and 20 µl to 30 µl and 10 µl, respectively. The robotic extraction was performed with robotic pipetting machine Qiacube (Qiagen). The same protocol was followed up until step 6, where supernatant was transferred to a separate tube and later loaded into the Qiacube for further procedure. All eluates were stored in -20°C before performing quality control (QC) on them. Before continuing with the synthetic miRNA of interest, the optimization of the method was done to ensure that the technique and the method was working (Figure 2).

Quality control

QC of the RNA eluates included the quantity check on the Qubit (Life Technologies) and Qubit miRNA assay kit (ThermoFisher Scientific), and quality using DS11 Spectrophotometer (Denovix).

RT and qPCR optimization

The optimization step included the reverse transcription or cDNA synthesis and qPCR steps. All the preparation was performed according to the Two-tailed RT-qPCR protocol (TATAA Biocenter) with adjusted volumes for each component (Table 1, Table 2). The synthetic miRNA used for the optimization step and for the rest of the project were different, as well as the flow of the work (Figure 2).

Synthetic miRNA miR-223 (Integrated DNA Technologies) in volume of 4 µl and concentration of 10^{13} copies/µl underwent the reverse transcription (Table 1) and later serially diluted in ten-fold from 10^{13} to 10^3 copies/µl. Concentrations from 10^9 to 10^4 copies/µl was used for the further qPCR run and for constructing a standard curve. Two spiked positive control samples and two non-spiked samples were run at the same time to see whether the samples would amplify. AriaMX was the program of use for the qPCR run and a pre-made RT program was used in this experiment.

Two-tailed reverse transcription

The RNA eluates were controlled for quality and right after prepared for the cDNA synthesis using the TATAA GrandScript cDNA FreePrime kit- protocol (TATAA Biocenter) with few changes in volumes according to Table 1.

Table 1. Components of RT reaction mix.

Component	Volume (µl)
GrandScript FreePrime Reaction Mix (5X)	2
GrandScript RT Enzyme	0.5

Two-tailed Primer (10μM)	2.5 (0.2 μM)
GSP Enhancer (10X)	1
Nuclease-free water	-
RNA template (1μg-10pg)	4
Total	10 μl

Two-tailed RT - qPCR

The two-tailed qPCR was performed according to the two-tailed RT-qPCR protocol (Table 2) in the AriaMX software (Agilent Technologies). The additional component, ROX dye, was added separately. When every component was mixed, each sample was plated in triplicates on the qPCR 96-well plate (ThermoFisher Scientific). Together with samples, a no-template control, also negative control (NTC), as well as no reverse transcriptase (-RT) were prepared and run in triplicates on the plate. The plate was vortexed and centrifuged for 30 sec. The plate was later run on the AriaMX qPCR (Agilent Technologies).

Table 2. Components of the qPCR reaction mix.

Component	**Volume (μl)**
TATAA SYBR® GrandMaster® Mix (2x)	5
Two-tailed forward primer (10μM)	0.2
Two-tailed reverse primer (10μM)	0.2
Nuclease-free water	2.4
ROX Reference dye (500nM)	0.2
cDNA	2
Total	10 μl

Standard curve and absolute quantification

The absolute quantification and standard curve analysis were performed from the qPCR run of the diluted samples of miR-223 and miRSeps-3 synthetic miRNAs. The prior synthesized cDNA from miRNA, was serially diluted in nuclease-free water in ten-fold from concentration 10^{12} to 10^{3} copies/μl. 10^{11} to 10^{3} copies/μl of miRSeps-3 and 10^{9} to 10^{4} copies/μl of miR-223 were used to plate them in triplicate. The absolute quantification on the unknown samples was performed using the miRSeps-3 standard curve, where the values of unknown samples were compared to the standards.

Statistical analysis

The statistical analysis was performed using SPSS (IBM SPSS, Version 28). Before the comparison of the data, The Shapiro-Wilk test of normality was performed on both concentration and purity values to detect and remove any outliers and see whether the distribution of the data was normally distributed. The Independent samples T-test with Levene's test for equality of Variances was performed on all data, where two sample groups were compared. The significance level on all tests was set to p=0.05. Descriptive statistics were performed on all sample groups. The standard curve and absolute quantification were done using Microsoft Excel Version 2204 (Microsoft).

Results

Quality control

The eluates, spiked with miRSeps-3 and non-spiked were measured for miRNA concentration, using the Qubit 4 (Thermofisher Scientific) and absorbance ration A260/230; A260/280 using DS11+ Spectrophotometer (Denovix). The raw data of all eluates are shown in Appendix (Table I, II, III, and IV). Out of 12 non-spiked samples that were manually extracted, 6 of them were out of range due to low small RNA, including miRNA, concentration, it was decided to put those samples' values to a constant of 0.04 and include them anyway, obtaining the constant with help of the limit of quantification (LOQ) equation:

$$\frac{LOQ}{\sqrt{2}} \rightarrow \frac{0.05}{\sqrt{2}} = 0.04\, ng/\mu l$$

However, all other samples could be measured for concentration.

Descriptive statistics

IBM SPSS was used to obtain the mean, median and Standard Deviation for the quantity and purity of the samples (Table 3; Table 4). Descriptive statistics were chosen to be performed on all four groups, i.e., manual spiked-in, manual non-spiked, robotic spiked-in, and robotic non-spiked, however, further statistical analyses were performed on two groups, manual and robotic extraction groups, where spiked and non-spiked samples were combined.

Table 3. Descriptive statistics for small RNA concentration within all groups.

Sample Group	Mean	Median	Standard Deviation
Manual spiked-in (n=12)	1.18	1.02	0.46
Manual non-spiked (n=12)	0.24	0.15	0.24
Qiacube spiked-in (n=12)	2.46	2.30	0.70
Qiacube non-spiked (n=12)	2.21	2.30	0.46

Table 4. Descriptive statistics for small RNA absorbance at A260/280 within all groups.

Sample Group	Mean	Median	Standard Deviation
Manual spiked-in (n=12)	1.55	1.29	0.73
Manual non-spiked (n=12)	1.73	1.51	0.80
Qiacube spiked-in (n=12)	0.63	0.62	0.12
Qiacube non-spiked (n=12)	0.66	0.62	0.12

Statistical analysis

Statistical tests were performed on all samples, combining spiked with synthetic miRSeps-3 and non-spiked ones, in order to see whether manual extraction or robotic one will give a better yield of small RNA in quality and quantity. The Shapiro-Wilk test of normality was performed to see the normality of distribution as well as to remove any outliers.

When performed on the small RNA concentration data and purity data, the Shapiro-Wilk test showed no significant departure from normality, as for concentration $W(24)=0.91$. $p=0.06$ for the manual extraction group, $W(23)=0.97$, $p=0.74$ for Qiacube extraction group and purity $W(22)=0.97$, $p=0.64$ for manual extractions, $W(24)=0.91$, $p=0.05$ for Qiacube extractions. The test indicated that the data was normally distributed, hence the independent-samples T-test was chosen to perform.

The independent-samples T-test with Levene's test for equality of variances was performed in order to see if there will be any statistically significant difference in variance of quantity and quality of miRNA between the manual and robotic extractions. The p-value was set to 0.05 and the hypotheses were stated:

H0=There is no significant difference between the groups (p>0.05)
H1= There is a significant difference between groups (p<0.05)

As a result, the test showed no significant difference in variance (p=0.122) in concentration between the manual and robotic extractions, while there was a significant difference in variance (p=<0.001) for the absorbance between the manual and robotic extractions. When comparing means between the groups, there was a significant difference (p=<0.001) for both quantity and quality. The group statistic with the Standard error mean and SD is shown in Table 5.

Table 5. The group statistics, where N is the number of samples. The spiked and non-spiked samples are mixed together for the statistical test, due to only the extraction type being analyzed.

	Sample group	**N**	**Mean**	**Std.Deviation**	**Std. Error Mean**
Concentration	Manual	24	0.71	0.60	0.12
	Qiacube	23	2.25	0.42	0.09
Purity	Manual	22	1.44	0.30	0.06
	Qiacube	24	0.65	0.12	0.02

Optimization

Due to niether spiked or non-spiked samples getting amplification in the optimization step, only a standard curve of miR-223 ten-fold serial dilutions (10^9 → 10^4 copies/µl) was generated. The curve (Figure 3) gave efficiency of 88%, efficiency of the curve was calculated using the equation:

$$E = 10^{\frac{-1}{m}} \rightarrow E\% = (E - 1) \times 100$$

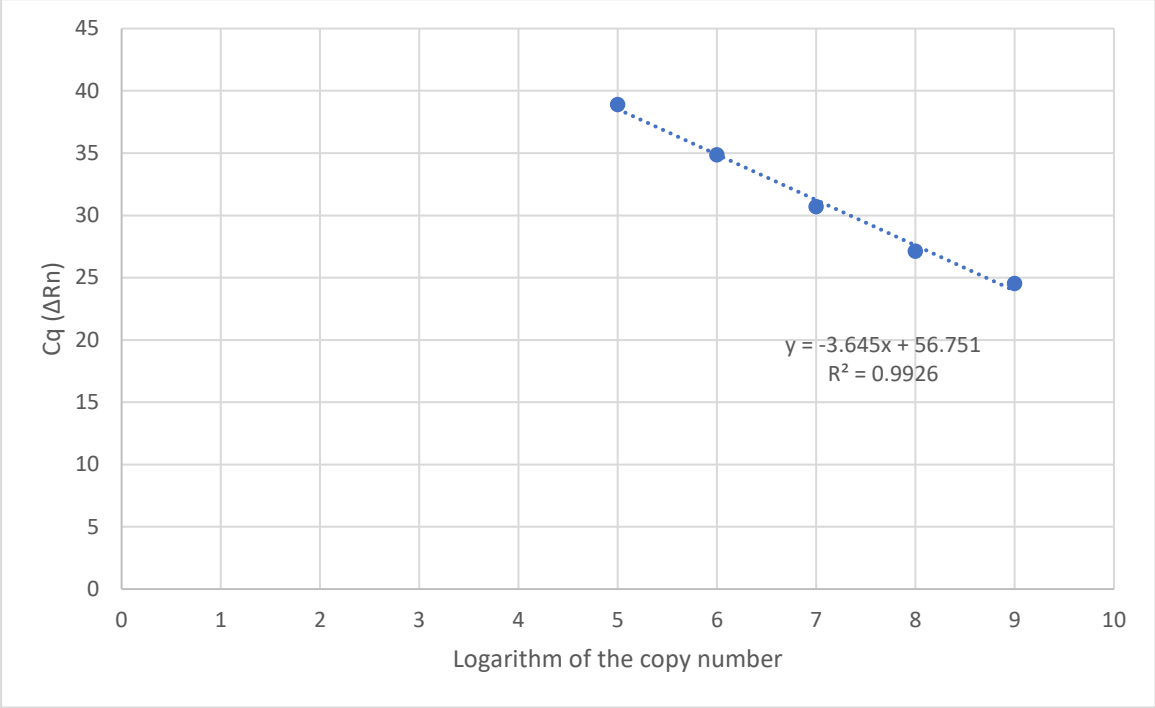

Figure 3. The optimized standard curve for the synthetic miR-223.

Two-tailed RT-qPCR

Both spiked with synthetic miRSeps-3 and non-spiked samples showed amplification apart from one sample. The no-template control (NTC) on the other hand also had amplification. One point (10^9 copies/µl) from the miRSeps-3 standard curve was taken in order to use it as an inter-plate calibrator (IPC). -RT did not show any amplification.

Absolute quantification

It was decided to proceed with generating a standard curve from the miRSeps-3 synthetic miRNA executing with the same method as it was done with synthetic miRNA miR-223, previously. The serial dilution of miRSeps-3 got amplified and a curve was generated with the efficiency of 100%, correlation efficient (R^2)of 0.998 and a slope of -3.307. The standard curve was plotted in Excel (Figure 4).

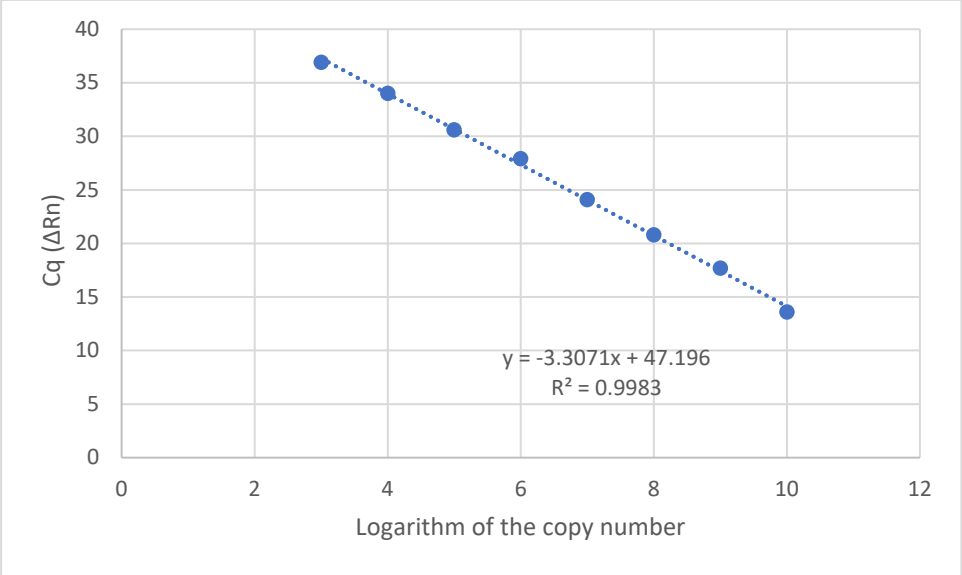

Figure 4. Optimized standard curve for miRSeps-3, with correlation efficient R^2 of 0.998, the slope of -3.307 and intercept of 47.196.

After the optimized standard curve, absolute quantification was performed on the unknown samples, which were non-spiked cDNA samples, to determine the copy number, with the help of the linear regression equation [$y = mx + b$]. To calculate the quantity of the unknown samples, the following equation was used:

$$N_n = 10^{\frac{Cq-b}{m}}$$

Quantified unknown samples (Table 6) were plotted into the standard curve (Figure 5).

Table 6. Absolute quantification of the unknown samples. Qext stands for Qiacube extraction, Ext stands for extraction. Samples used here were chosen due to them having the closest purity ratio to 2.0 from the raw data (Table III, Table VI, Appendix).

Sample	Cq	Log Concentration
Qext6	29.82	$10^{5.2}$
Qext7	32.43	$10^{4.5}$
Ext3	30.39	$10^{5.08}$
Ext5	30.45	$10^{5.06}$

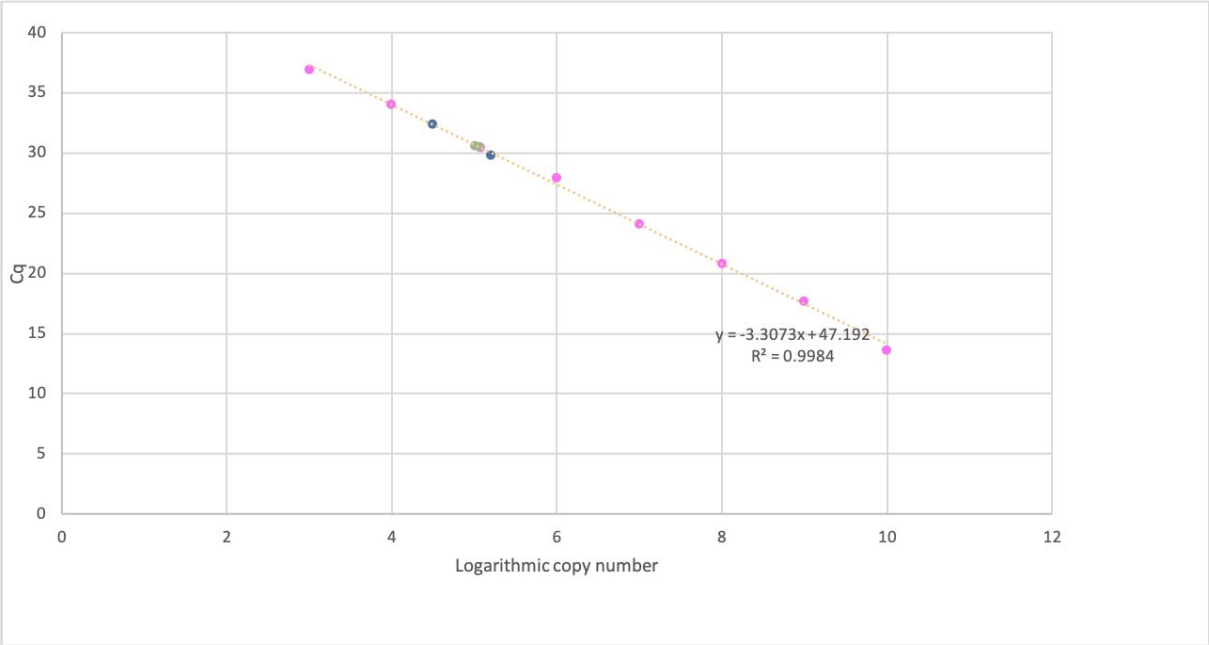

Figure 5. A standard curve with plotted-in quantified unknown samples (green for manual extractions and blue for Qiacube) and the dilutions of miRSeps-3 (pink).

Discussion

The project in general aimed to compare manually and robotically extracted small RNAs samples from healthy human blood plasma, using the mRNeasy Serum/Plasma Advanced Kit (Qiagen), for the concentration of small RNA, including miRNA, and purity of the same. When extracting samples manually, every step of the mRNeasy Serum/Plasma Advanced Kit protocol (Qiagen) was followed, only with a few changes in volumes of buffers and isopropanol, in order for the ratio to be 1:1 (mixture:isopropanol) according to the mRNeasy Serum/Plasma Advanced Kit protocol. On the other hand, the robotically extracted samples were prepared manually according to the protocol up until step 6, where the buffer volumes were adjusted. However, the Qiacube pipetting machine (Qiagen) could not by itself change the isopropanol volume, hence, the pre-programmed volume of 230 µl was added to the plasma samples to be extracted. The 230 µl comes from the machine initially being programmed to work with 200 µl of the plasma, while in this project only 100 µl was used, due to previous thesis workers (von Ehr, 2020) comparing the volumes and indicating that 100 µl is enough for the extractions in order to obtain readable results from concentration and purity control. The amount of added isopropanol, however, did not affect the miRNA concentration and purity results according to other students performing this comparison (Personal communications during project, 2022).

Before extraction of small RNA, all the plasma was divided into spiked and non-spiked categories. The spiked category was referring to the plasma spiked with synthetic miRSeps-3 (Integrated DNA Technologies) for the main experiment part and miR-223 (Integrated DNA Technologies), for the optimization parts of the project. The reason behind spiking the samples was for the later use of it during the qPCR, more specifically, to see whether the Two-tailed RT-qPCR method (TATAA Biocenter) is able to detect the specific synthetic miRNA and use it as a control, indicating that the method is working correctly and to be able to perform absolute quantification to quantify the amount of miRNA in non-spiked samples.

The quality and quantity of small RNAs (Appendix. Table I, II, III, and IV) of the eluates were measured with Qubit 4.0 Fluorometer (Life Technologies) and DS11 Spectrophotometer (DeNovix), respectively. The choise of using the Qubit for concentration quantification was because of a study Wright, de Silva, Purdie & Plain, 2020 did, where they showed the Qubit results being most consistent when comparing to other methods. The results showed that robotically extracted samples had statistically higher small RNA quantity, but lower quality, the absorbance at A260/280 (Table 3, Table 4). Even though the Qubit has the ability to detect the small RNA, including miRNA, in concentrations between 0.05 ng/µl and 100 ng/µl (Life Teachnologies), it does not really explain the difference in concentrations between two extraction methods (Das Gupta et al., 2020). Furthermore, all the samples' concentrations were on the "lower side" of the Qubit detection concentration range. Different concentration that would land on the higher end could have given different purity results. Another reason behind the difference in concentration between manual and robotic extractions could be the higher amount of isopropanol used in the robotic extractions. On the other hand, the sample size for each group (n=12) is too small in order to judge the whole method (Serdar et al., 2020). While planning the project, it was first decided to make more extractions than 12, however, during the method testing it was clear that there would be no time to make many extractions. The reason behind 12 extractions per group was that Qiacube is able to do 12 extractions at a time. To not use the machine too many times and save time, the sample size was decided to be kept at 12 and use Qiacube only two times. While analyzing the results, it was seen that a bigger sample size would probably give more precise and reliable results.

Furthermore, six samples from the manual non-spiked group had too low to count concentration. This does not necessarily mean that something is wrong with the samples or that the miRNA is non-existent there, but there are some limitations to the Qubit machine (ThermoFisher Scientific,

2021). The Qubit 4.0 Fluorometer can read samples that have a concentration of at least 0.05 ng/µl (Thermofisher Scientific, 2021), which can be the reason why the samples were not detected. However, as mentioned above, this does not mean that there is no existing small RNA as well as miRNA in the samples, hence the specific samples were chosen to keep and later use in the two-tailed RT-qPCR for the detection and quantification. To be able to keep the samples and use them in the statistical analysis, their concentration values were set to 0.04, which is a constant of the equation LOQ /√2. The method is called single imputation and is one of the methods used to analyse the data that is below the limit of quantification (LOQ) (Succop, Clark, Chen and Galke, 2004; Harel et al., 2014).

The purity of small RNAs control, that was performed using DS11-Spectrophotometer, showed the purity of the samples being higher for manual extractions (Table 4). Both absorbances at A260/280 and A260/230 were measured, but only A260/280 was used for statistics and further analysis, because of bad planning and eventually facing the lack of time. For the quality difference, once again, the extra added amount of isopropanol in robotic extractions could be the reason for the different results (Moret et al., 2013), but nothing pointed to that, compared with other students in personal communication during the project work. On the other hand, even though manual extractions had higher and better purity values than robotic ones, they still did not reach the desired 2.0. The reason behind that could, for instance, be the required amount of concentration for accurate measurements (Koetsier and Cantor, 2019). DS-11 considers samples with a concentration <10 ng/µl possibly too diluted and close to lower detection limit. None of the samples reached 10 ng/µl in concentration, which could possibly be a reason behind the wrong measurements (Koetsier and Cantor, 2019). In a study Kalmár et al (2014) did, they examined the difference in quality and quantity between manual and robotic extractions and results showed the clear advantage of manual extraction method.

The low purity, in general, could be explained by contaminants or low RNA concentration (Koetsier and Cantor, 2019). Manual extractions values were not that much below the desired purity ratio (2.0) in comparison with robotic ones. Possible organic contaminants could be phenol and guanidine, which are common in isolation protocols (O'Neill et al., 2011). However, phenol was not present in the buffers from the kit, which lead to a conclusion, that either another contaminant was present, the guanidine led to incorrect readings, or the reason is not the contamination. Another possible explanation could be the contaminated blank sample. Low purity values (A260/280) can also be explained by protein contaminations (O'Neill et al., 2011). The quality control results, however, probably did not affect the downstream application of qPCR, because the extracted samples were put into comparison with "pure" synthetic miRNA when compared with standards. In other words, there was probably a sufficient amount of small RNA for the process to work (Li, Dov, Mauro and Williams, 2015). However, this can be considered for future projects when doing the quality control of the extractions done with the same kit. In another study performed by (Mathieson et al., 2018) it was also shown that manual extraction was preferred over the robotic ones due to better results.

A major part of the project was to see whether it would be possible for the two-tailed RT-qPCR method to amplify the synthetic miRNA miRSeps-3 and later to be able to quantify the amount of miRSeps-3 in unknown samples using the standard curve and absolute quantification. The optimization of the standard curve process was made with plasma that was spiked with synthetic miR-223, due to it being known for working (Marinkovic, 2021). The plasma was spiked with miR-223 in 10^5 and 10^6 copies/µl and extracted both manually and robotically, each extraction type per sample, according to the mRNeasy Serum/Plasma Advanced Kit protocol. Together with spiked plasma, a no reverse transcriptase (-RT), no template control (NTC), and a serial dilution of miR-223, which would act as standards, were prepared following the same protocol and were used as negative controls. When examining results from the qPCR run, it could be seen that neither control samples or -RT and NTC got any amplification, while the standards amplified well. The standard curve was later plotted in Excel and slope, efficiency, and R^2 were calculated.

Ideally, when making a standard curve from a 10-fold serial dilution, the slope should be -3.33 when the efficiency is 100% (Svec et al., 2015). The miR-223 standard curve had a slope of -3.6, while the efficiency was around 88%. When plotting the curve, only five out of six points were included because one sample did not amplify. An attempt to increase the efficiency was made by removing one additional point, leaving only four points for plotting the standard curve. The efficiency went up to 100% and the slope went down to -3.307. Although the efficiency between 90% and 110% is considered acceptable, only four points are way too little, because a difference in the efficiency can lead to a big difference in later input (Bivins et al., 2021). Since the efficiency changes a lot in percentage, it was decided to keep all five points and continue with 88% efficiency of the standard curve. The standard curve was re-made several times following the same protocol and steps, however, the results were not improved. The reason behind the gotten results can be the inefficiency or degradation of either RT or qPCR primers (ThermoFisher Scientific, n.d.), because of the freezing-thawing process. In two studies, made by Dzung et al, 2021 and Cuthbertson et al., 2015, they studied the freezing-thawing cycles of biological materials, which resulted in degradation of the quality. These studies were not exactly about primers, but it could possibly be applied to the primers, as well. Another factor could be possible organic contamination, such as spores in the air, that could have been acquired during the aliquotation of the fluid from the original bottles. The same buffers and primers were used by every project student several times. The aliquotation of the needed amount in the beginning of the project was not possible due to several changes in the plan. After several attempts, it was decided to continue the project with the miRNA of interest, miRSeps-3. This decision was taken based on the previous thesis workers and previous studies that worked with miR-223 and got accepted results (Marinkovic, 2021), which lead to a small conclusion that the problem was not laying in the miRNA, and not in the technique, since the miRSeps-3 standard curve worked out well, but most likely in the materials. However, proceeding with the rest of the experiment was not affected by that.

Once moved on to the "real" standard curve, the same exact process was followed to obtain it. The plasma used for this part of the experiment was spiked with miRSeps-3 in concentrations -10^4, -10^5, and -10^6 copies/µl, with four samples per concentration and extraction method. After the qPCR run, the standard curve was plotted in Excel and efficiency was calculated to be 100%, slope -3.31, and R^2 0.999. The results of the standard curve fell into the range of being considered good (Bissels et al., 2009). The first run was only made to construct a working curve for later usage in the quantification of the unknown concentration in non-spiked samples. Since the standard curve was accepted as a good working curve, one point from it (10^9) was taken from it to use as an inter-plate calibrator. An inter-plate calibrator serves as a control to decrease the variations between different plates (Rieu and Powers, 2009).

The results from the second plate, which contained the IPC, NTC, -RT, spiked samples (n=2), and non-spiked ones (n=4), showed the amplification (Cq range 32.51 to 34.71) (Appendix, Figure I) of all samples, with exception of one spiked samples triplicate. However, this means that NTC also got amplified, which indicated that either contamination was present, or some part of the method was done wrongly. It was decided not to re-run the plate, because lack of time, and proceed with absolute quantification of the unknown (non-spiked) samples, because the NTC on the previous plate did not amplify (Appendix, Figure II), which can be explained by an unwanted contaminant in one of the buffers while preparing the last plate.

Subsequently, the unknown (non-spiked) samples were plotted into the standard curve to obtain the quantity of miRNA in the samples (Figure 5), using the absolute quantification. Absolute quantification is seen as one of the standard methods of quantifying PCR results (Boulter et al., 2016). The unknown samples' mean Cq were taken and quantified with the help of linear regression equation and later compared with the standard curve. As it could be seen (Figure 5), there was no big difference in the log copy number of all unknown samples. By comparison to the standard curve, it could be seen that the unknown samples were successfully quantified.

The statistical analysis contained both descriptive statistics and comparing the mean of the data between different groups. The statistical tests performed were not relevant when it comes to evaluate the Two-tailed RT-qPCR method, instead they were performed to see which extraction method would give better results of the samples (Table 5). Even thought there was a difference in both concentration and purity between the groups, it did not affect the further performance. The statistical results showed significant difference in both concentration and purity between extractions, however, two other studies (Cornelissen et al., 2017; Sharma et al., 2022) when comparing the RNA extraction methods did not show significant difference between methods. It cannot be to 100% fairly compared with these studies, due to them using different kits as well as extraction of the RNA, not only small RNAs including miRNAs. On the other hand, the process is very similar.

Ethical aspects, gender perspectives and future studies

In this study, the blood was taken and used from self-assessed donor, that beforehand got all information. No personal information was gathered, neither anything about the donors was published anywhere. Since everything remained confidential, no ethical approval was required for this part of experiment.

This project is a part of the bigger studies called "Future diagnosis of Sepsis", which has a goal to create an easy and fast way to diagnose sepsis on early stage, by ideally creating a multimarker panel, that would decrease the mortality, and usage of drugs and antibiotics, that can potentially lead to more antibiotic resistance, which already is an issue. Another issue known is the gender inequality in healthcare. It has shown, that patients equally ill got different assist and help depending on their gender (Samuelsson, Sjöberg, Karlström, Nolin and Walther, 2015). It has also been reported that patients with sepsis and septic shock also got different care in emergency department, depending on their sex (Sunden-Cullberg, Nillson and Inghammar, 2020). The hope is that the future multimarker panel may eliminate these inequalities.

This specific project had a goal of evaluating a specific synthetic miRNA biomarker too see whether it would be detected and if there was a possibility to quantify it. The extraction was performed using the miRNeasy Serum/Plasma Advanced Kit, which is a kit that can hopefully be used in clinical laboratories in the future. This project also aimed to see whether Qiacube pipetting machine was a good alternative for extraction method.

The hope is that this project would help scientist in the future when it comes to choosing the extraction methods for the miRNA that is going to be a part of the future multimarker panel, which would help a lot of patients to be saved before the sepsis becomes fatal.

Summary of weaknesses of the study

This thesis project had several weaknesses that, if avoided, could give different results. The usage of Qubit as a method of concentration measurement. A study compared Qubit with other concentration measurement methods, and even though the Qubit may not be the most precise method, it had the best consistency (Wright, de Silva, Purdie & Plain, 2020). Using another method could have shown different concentration results. Also, what should be taken in consideration is that small RNA (that Qubit is able to detect) do not consist of much miRNAs (Garcia-Elias et al., 2017) and that is why the results in this project are not only for pure miRNAs.

The sample size used to "judge" a whole extraction method was actually too small to say for sure that one method is better than another (Serdar et al., 2020).

Conclusion

As a conclusion, the research about the usage of miRNAs as biomarkers is an important field and has a lot of potential (Sandquist and Wong, 2014). However, the usage of miRNAs as biomarkers is good when the whole method, from extraction, to quantification is optimized. The project tested two different extraction methods in order to see which method would yield higher in small RNA concentration and purity. Based on the results, both extraction methods still require optimization to reach its full potential. This study also tried out the two-tailed RT-qPCR method to see if it was capable to detect miRNAs from human plasma. The method showed good to excellent results in detecting both synthetic miRNAs, more specifically miRSeps-3, and miRNAs from samples with no spike-in controls.

Moreover, the miRNA extraction and detection can be applied in different research fileds and not being limited to the sepsis project. Further research and optimization of similar projects have good potential to reach the main goal of early diagnosis of sepsis.

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Appendices

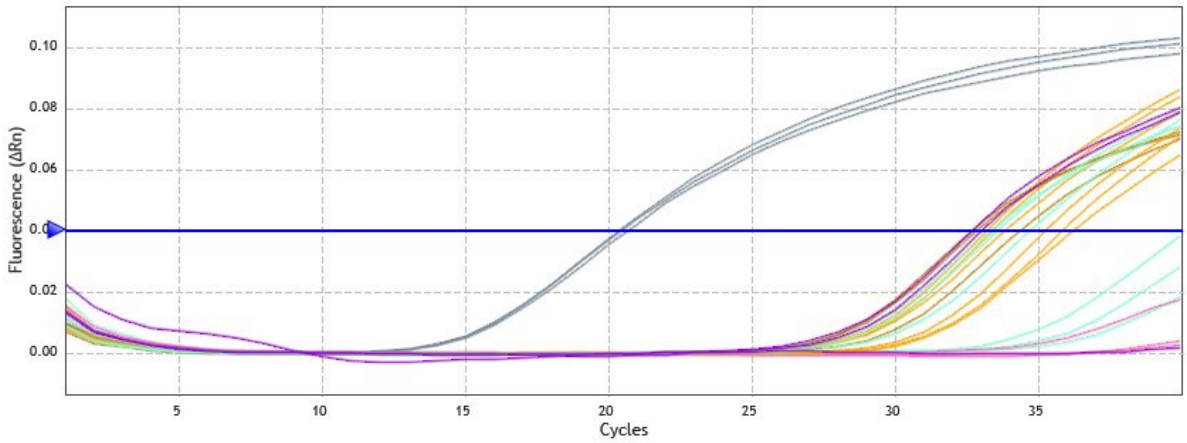

Figure I. Amplification plots for the spiked samples (n=2), non-spiked (n=4), inter-plate calibrator (grey) and NTC (blue). The NTC showed some amplification, one triplicate sample did not show any amplification (pink).

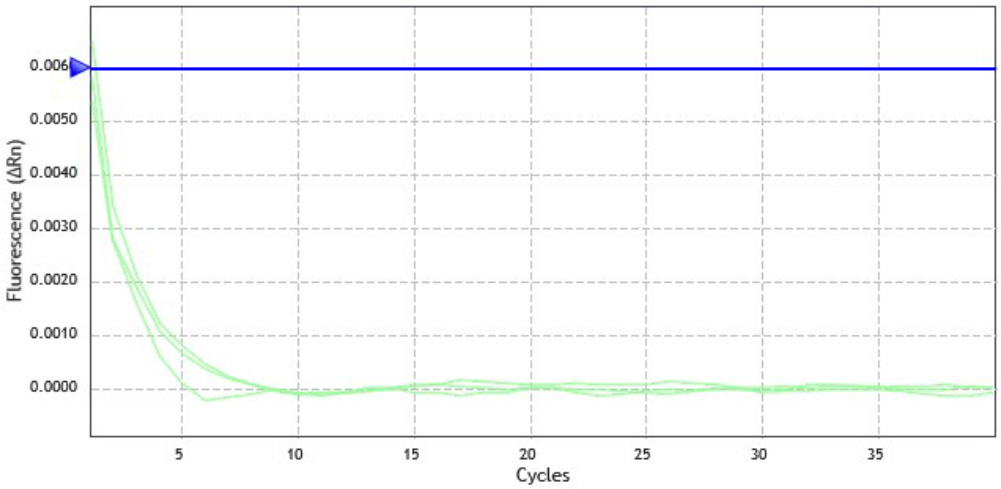

Figure II. The amplification plot of the NTC on the plate containing the standards. Standards not shown to show more clear picture of the NTC amplification.

Table I. Concentration and absorbance at 260/230; 260/280 of manually extracted spike-in with miRSeps-3 samlpes.

Sample name	Concentration ng/µL	A260/230	A260/280
Ext sp 1	0.802	0.185	2.103
Ext sp 2	1.59	0.431	1.323
Ext sp 3	1.36	0.345	1.247
Ext sp 4	2.23	0.081	1.249
Ext sp 5	1.01	0.058	1.515
Ext sp 6	0.650	0.092	1.434
Ext sp 7	0.730	0.318	3.669
Ext sp 8	1.04	0.310	1.157
Ext sp 9	0.888	0.123	1.600
Ext sp 10	1.01	0.012	1.221
Ext sp 11	1.15	0.292	1.159
Ext sp 12	1.67	0.227	0.886

Table II. Concentration and absorbance at 260/230; 260/280 of robotically extracted with Qiacube spike-in with miRSeps-3 samples.

Name	Concentration ng/µL	260/230	260/280
QExt sp 1	1.90	0.166	0.881
QExt sp 2	2.06	0.109	0.685
QExt sp 3	2.33	0.115	0.695
QExt sp 4	2.61	0.107	0.667
QExt sp 5	2.26	0.156	0.816
QExt sp 6	1.97	0.071	0.497
QExt sp 7	1.89	0.089	0.542
QExt sp 8	2.02	0.078	0.515
QExt sp 9	2.42	0.081	0.524
QExt sp 10	3.19	0.101	0.637
QExt sp 11	2.47	0.095	0.601
QExt sp 12	4.35	0.088	0.537

Table III. Concentration and absorbance at A260/A230; 260/280 of manually extracted non-spiked samlpes.

Sample name	Concentration ng/µL	260/230	260/280
Ext 1	OOR	0.063	1.496
Ext 2	0.440	0.055	1.323
Ext 3	0.480	0.338	1.761
Ext 4	OOR	0.280	1.352
Ext 5	0.280	0.027	1.844
Ext 6	OOR	0.240	1.959
Ext 7	OOR	0.255	4.111
Ext 8	0.625	0.170	1.136
Ext 9	0.250	0.202	1.108
Ext 10	OOR	0.644	1.438
Ext 11	0.594	0.642	1.531
Ext 12	OOR	0.050	1.732

Table IV. Concentration and absorbance at 260/230; 260/280 of robotically extracted with Qiacube non-spiked samples.

Sample name	Concentration ng/µL	260/230	260/280
QExt 1	2.68	0.090	0.585
QExt 2	2.79	0.104	0.698
QExt 3	2.29	0.095	0.628
QExt 4	1.45	0.110	0.646
QExt 5	2.32	0.101	0.611
QExt 6	1.93	0.180	0.959
QExt 7	1.45	0.120	0.802
QExt 8	2.67	0.089	0.559
QExt 9	2.62	0.107	0.610
QExt 10	2.06	0.097	0.589
QExt 11	2.39	0.075	0.498
QExt 12	1.91	0.105	0.727