Degree project

UNIVERSITY OF SKÖVDE

EXTRACTION OF MIR-223 FROM HUMAN BLOOD PLASMA AND QUANTIFICATION USING THE TWO-TAILED RT-QPCR AND ABSOLUTE QUANTIFICATION

Bachelor Degree Project in Bioscience, 30 credits

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Abstract

Sepsis is a very dangerous and life-threatening disease that develops when the body's reaction to infection causes damage to the body's tissues and organs. It is difficult to diagnose it and it develops fast leading to a high mortality rate. Current methods rely on blood culturing and multiple biomarkers, such as C-reactive protein and procalcitonin, that take too long to produce results. A possible solution to this problem lies in specific biomarkers such as microRNAs, which are small non-coding single stranded RNA molecules that contain around 22 nucleotides and have a big role in regulating gene expression. Being specific biomarkers for particular disease makes microRNAs promising biomarkers for sepsis. The aim of the project was to optimize the process from extraction to quantification of microRNAs using the miRNeasy Serum/Plasma Advanced Kit-Qiagen Kit (manual) and to see if the Two-tailed RT-qPCR (TATAA Biocenter) technique could quantify the samples. Blood plasma from healthy donors was used for microRNA extractions and was separated into two categories- spiked-in samples and non-spiked samples. Spiked-in samples were spiked with a synthetic microRNA- miR-223 and served as a positive control. All samples were quantified using the absolute quantification and the Two-tailed RT-qPCR method (TATAA Biocenter). Quantification was successful for all samples showing that the method was optimized, parameters for optimization were within the wanted range, and quantifiable. More research is needed, however, the method has potential in becoming a simple and quick novel tool in diagnosing sepsis in the early stages and thus saving lives.

Abbreviations

SIRS	Systemic inflammatory response syndrome
SOFA	Sequential organ failure assessment
CRP	C-reactive protein
PCT	Procalcitonin
DNA	Deoxyribonucleic Acid
RNA	Ribonucleic Acid
miRNA	MicroRNA
qPCR	Quantitative Polymerase chain reaction
RT-qPCR	Quantitative reverse transcription Polymerase chain reaction
RT	Reverse Transcription
cDNA	Complementary DNA
QC	Quality control
NTC	No template control
-RT	No reverse transcriptase
SD	Standard deviation
Cq	Quantification cycle
R^2	Coefficient of determination

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Introduction

Sepsis

Sepsis is a very dangerous and life-threatening illness that develops when the body's reaction to infection causes damage to the body's tissues and organs (Salomão et al., 2019). This means that when the infection fighting processes malfunction it causes the body to react incorrectly and its organs to work inadequately and abnormally (Angus and van der Poll, 2013).

Typical symptoms of sepsis include fever, increased heart and breathing rate and within specific infections, like pneumonia, cough is frequent. Other indications may include low blood pressure, high blood lactate and low urine output (Seymour and Rosengart, 2015). A severe drop in blood pressure is associated with septic shock that can lead to organ shut down and death (Seymour and Rosengart, 2015). Various types of infection can cause sepsis. The most common type of infection is bacterial (Minasyan, 2019) but viral (Lin et al., 2018) and fungal infections could also lead to sepsis. The risk groups for this condition include very young children and older people as well as anybody with a weakened immune system (Frydrych et al., 2018). This means that people with conditions like diabetes, cancer, HIV and burns have a higher chance of developing sepsis (Liu, Mahale and Engels, 2019).

It is difficult to diagnose sepsis, firstly because it is similar to a variety of other diseases and secondly because it progresses very fast so by the time the condition has been identified the patient may already be gone. In the past, the existence of at least two systemic inflammatory response syndromes (SIRS) had to be identified in the body for the person to be diagnosed with sepsis, but a new method has been put to use (Bosmann and Ward, 2013; Kaukonen et al., 2015). The golden standard for diagnosing sepsis is still blood culture. A blood culture test identifies what type of infection is in the blood (Henriquez-Camacho and Losa, 2014), however, this test requires a larger volume of blood (10mL per bottle) to test, and can take several days to generate results, which could be fatal for the patient (Murray and Masur, 2012). A novel way of detecting sepsis was developed in order to speed up the diagnosis process. It was the shortened sequential organ failure assessment score (SOFA score). For the SOFA score to be an identifier of the disease the person had to have at least two out of three symptoms: increased breathing rate, low blood pressure and a change in the level of consciousness (Van der Woude et al., 2018).

A person diagnosed with sepsis requires immediate medical attention in the form of fluid replacement and antibiotics. If the intravenous fluids are not enough, medication is given in order to raise the blood pressure of the patient. Depending on the symptoms and infections, mechanical ventilators and dialysis may be given to patients with pneumonia and kidney problems, respectively. Another helpful way of diagnosing sepsis is with the use of biomarkers (Faix, 2013).

Biomarkers

Biomarkers are measurable biological markers of a biological condition (Califf, 2018). They are frequently used in many scientific fields, they are quantified and assessed in order to help analyze biological and pathogenic processes and to help pharmacological drug development. Biomarkers in medicine are relatively novel techniques that can be categorized into three groups according to their clinical functions. They are grouped as molecular, cellular and imaging biomarkers. All of those groups act as a helpful tool in choosing a perfect treatment (Canese et al., 2020).

They are sub-grouped into predictive, diagnostic and prognostic biomarkers. Predictive biomarkers predicts clinical outcomes and help in the selection of the ideal treatment. Diagnostic biomarkers narrow down the diagnosis, making them more specific for a patient and prognostic biomarkers provide the information about the patient's aftermath (Jekarl et al., 2019). Biomarkers

in sepsis have become a new potential diagnostic tool as they may allow for an earlier diagnosis than other techniques (Mustafić et al., 2018). Several applications have been established when it comes to biomarkers in sepsis, this includes diagnosis of infection, prognosis of the disease and guidance when it comes to medical treatment (Sandquist and Wong, 2014).

Biomarkers in sepsis

C-reactive protein (CRP) and procalcitonin (PCT) are currently the most used biomarkers to recognize sepsis (Ljungström et al., 2017) but other biomarkers such as microRNAs (miRNA) and pro-inflammatory cytokines are being examined as potential biomarkers for sepsis (Dumache et al., 2015). It has been noticed that the combination of pro- and anti-inflammatory biomarkers in a multimarker panel help recognize patients who are starting to develop severe forms of sepsis before the organs malfunction and sepsis progresses too far. Sepsis biomarkers have the potential to provide much more information than other techniques and thus help diagnose the condition earlier, recommend and monitor the effectiveness of the prescribed treatment and change it on time if necessary (Faix, 2013).

MicroRNAs

As mentioned before miRNAs are novel potential biomarkers of sepsis and were used for this project. The detection from spiked and non-spiked human blood plasma was performed (miRNA-223 was used for spiking). MiRNAs are small non-coding single stranded RNA molecules that contain around 22 nucleotides and have a big role in regulating gene expression (Wahid et al., 2010). Some other roles that are associated with miRNAs are cell proliferation, cell death, cell development and metabolism (Wahid et al., 2010). Most of the miRNA are transcribed from DNA into primary miRNA, processed into precursor miRNAs from which mature miRNAs form. MiRNAs can be secreted into extracellular fluid from which they are transported via vesicles or binding proteins. Extracellular miRNAs have a function of a chemical messenger and mediate cell-cell communication, they are released into the body fluids and have a potential as biomarkers in a number of diseases (Benz et al., 2016).

Some of the diseases that miRNAs have been implicated to be involved include cancer (Peng and Croce, 2016) cardiovascular diseases, inflammatory diseases (sepsis), liver diseases (hepatitis) and other (Ardekani and Naeini, 2010). The development of miRNAs as biomarkers is steadily growing in hopes that their development will potentially provide better treatments for diseases.

Aim

The aim of the project was to optimize the process from extraction to quantification of micro-RNA (miRNA), recognised as a potential biomarker of sepsis, from human blood plasma. More specifically if the miRNeasy Serum/Plasma Advanced Kit- Qiagen Kit (which was done manually as opposed to the use of QIAcube that was used by another student) was specific enough to be able to extract miRNAs and if the qPCR technique could quantify the non-spiked samples. More accurately, if the Two-tailed RT-qPCR was sensitive enough to be able to quantify, with the help of absolute quantification, the non-synthetically spiked elutes and what their concentrations would be. This project is indirectly connected to the "Future diagnostics of sepsis (University of Skövde, n.d.)" project and is done in order to help future research projects to develop a simple and quick diagnostic kit for early detection of sepsis.

Research question

As for the research question, it is connected to the aim. The question is if the manual Qiagen kit can extract miRNAs from human blood plasma, or will only the detection of spiked synthetic miRNAs be possible. Another question that could be asked is how pure are the potential identified miRNAs from human blood plasma (non-spiked) and what their concentrations are using the said

kit. As it is difficult to extract microRNAs from bodily fluids, and this project worked with a volume of 100µL of human blood plasma, this was one of the limitations of the project. It was essential to work accurately and analyze if steps of extraction to quantification needed additional optimization in order to achieve the quantification of the non-spiked samples successfully. The objectives of the project were:

- To perform miRNA extractions from plasma, manually, with the miRNeasy Serum/Plasma Advanced Kit (Qiagen)
- Measure the quality and quantity of the RNA elutes
- Perform a Two-tailed RT-qPCR
- Quantify the miRNA elutes using absolute quantification

Materials and methods

Ethical consideration

The study about sepsis (Ljungström et al., 2019) had an ethical approval from (the Regional Ethics Committee in Gothenburg, no. 376–11) all the patients participating in blood donation have signed informed consent and were informed of the project aim and strategy. However, the thesis project used blood extracted from self-assessed healthy adult volunteers. More specifically their blood plasma was used. These self-assessed healthy adults willingly donated their blood for the research. So, no ethical approval was needed for the project.

Plasma samples

Plasma was taken from the self-assessed healthy volunteers and stored in a -80˚C freezer until it was used for this project. Human blood plasma frozen in the -80˚C freezer was thawed for 14 minutes in a 37˚C waterbath (VWR) each time it was taken out of the freezer. 97.4µL of the plasma sample was taken and spiked with 2.6µL of the synthetic miR-223 (Integrated DNA Technologies).

Extraction of total RNA

The extractions were performed manually according to the miRNeasy Serum/Plasma Advanced Kit (Qiagen). Only modifications to the protocol were using 100µL of blood plasma instead of 200µL, buffer RPL- 30µL (for 100µL of plasma) and buffer RPP- 10µL (for 100µL). The extractions were performed in a span of three days and the spiking-in occurred before the protein precipitation step in the miRNeasy Serum/Plasma Advanced Kit (Qiagen; step 3 before the addition of buffer RPP.) Each day four samples of one of the three spike-in copy numbers/µL - 10^7, 10^6 and 10^5 copies/µL were extracted in order to have four samples per each copy number/µL. The 10^7, 10^6 and 10^5 copies/µL were gotten by making a serial dilution of miR-223 in nuclease-free water from the initial concentration of 10^{13} copies of miR-223. Spiked-in elutes served as a positive control to non-spiked elutes later on during the qPCR runs. Ensuing the work with spiked-in plasma samples, work with non-spiked plasma samples was performed following miRNeasy Serum/Plasma Advanced Kit (Qiagen). The exact modifications to the protocol as for the spiked-in samples were implemented, only difference between the spiked-in plasma samples and the non-spiked plasma samples was that there was no synthetic spike-in in the non-spiked samples.

Quality control of the extractions

Once the extraction from plasma samples was complete and the elutes were obtained (for both spiked-in and non-spiked plasma samples), quantity of the miRNA was analyzed using the Qubit 3.0 Fluorometer Manual (Life Technologies) and Qubit microRNA Assay Kit by detecting fluorescence signals and converting them into an RNA concentration measurement. Quality was analyzed using the DS11+ Spectrophotometer Nanodrop (DeNovix). Once the quality control (QC)

was performed, elutes were stored in the -80˚C freezer until they were needed for RT and qPCR later on.

Two-tailed cDNA synthesis

Once quality and quantity have been analyzed, reverse transcription or cDNA synthesis of the samples was performed using the TATAA GrandScript cDNA FreePrime Kit-User Manual v.1.0 (TATAA Biocenter) protocol. The cDNA synthesis was performed for all elutes (spiked-in and non-spiked), for the serial dilution of miR-223 and the no reverse transcription (-RT) of all plasma elutes (both spiked-in and non-spiked). The total reaction volume was 10µL per reaction (Table 1).

The two-tailed primers (TATAA Biocenter) were provided at the concentration of 10µM and were diluted in nuclease-free water to the final concentration of 0.2µM. The amount of RNA template added to RT reactions varied. Negative control,-RT, of all elutes (spiked-in and non-spiked) did not contain the RT enzyme; instead the volume was compensated with nuclease-free water. The cDNA synthesis was run in the Thermocycler (Biorad) for 45 minutes at 42˚C, then 5 minutes at 85˚C and finally kept at 4˚C.

Table 1. Components of the RT reaction from TATAA GrandScript cDNA FreePrime Kit

Components	Volume (µL)
GrandScript FreePrime Reaction mix (5X)	2
GrandScript RT Enzyme	0.5
Two-tailed primer (10µM)	2.5 (0.2µM)
GSP Enhancer (10X)	1
Nuclease-free water	-
RNA template (1µg-10pg)	4
Total	10µL

Two-tailed RT-qPCR

Following the cDNA synthesis, Two-tailed reverse transcription quantitative real-time polymerase chain reaction (Two-tailed RT qPCR) was performed following the TATAA SYBR® GrandMaster® Mix- User Manual v.1.0 (TATAA Biocenter) and was used as the method for the detection and quantification of miRNA. The components of the qPCR are shown in Table 2. Another alteration made in the protocol was the addition of ROX (Biotium). All cDNA samples (from both spiked-in and non-spiked elutes) were run in triplicates. The primers (forward and reverse; TATTA Biocenter) were provided at the concentration of 10µM, so 0.2µL was used per primer per reaction to get a working concentration of 0.4µM. The amount of cDNA template was 2µL for all the samples except the no template control (NTC), a negative control in the qPCR reaction, which does not contain any template. The samples were loaded on the 96-well PCR plate (Applied Biosystems), sealed using a sealing film, and the plate was placed into the 7300 Real Time PCR System (Applied Biosystems) and ran according to the protocol on the following cycling program- 30 seconds at 95˚C, 40 cycles consisting of: 5 seconds at 95˚C, 15 seconds at 60˚C and 30 seconds at 72˚C. The results were obtained afterwards.

Table 2. Components and volumes of the qPCR reaction

Components	Volume (µL)	Final concentration/amount
TATTA SYBR GrandMaster Mix (2X)	5	1X

Two-tailed Forward primer (10µM)	0.2	0.4µM
Two-tailed Reverse primer (10µM)	0.2	0.4µM
Nuclease free-water	2.4	-
cDNA template	2	-
ROX (25µM)	0.2	500nM
Total	10	

Absolute quantification

A ten-fold serial dilution was prepared using the synthetic miR-223 (Integrated DNA Technologies) and nuclease-free water. The serial dilution had a range from 10^{12} to 10^5 copies/µL. The curve was constructed in Excel (2010) by plotting the dilution quantification cycles (Cq) and the logarithmic copy number/µL. In order to determine if the qPCR was optimized the coefficient of determination (R^2) and the amplification efficiency were computed. For the extracted plasma samples, absolute quantification was used in order to obtain the unknown concentration of the miR-223 in the non-spiked elutes.

Statistical analysis

Once the results were obtained, a statistical analysis was performed in the SPSS computer program (IBM SPSS) and Excel (Microsoft, 2010). Descriptive statistics like mean, median, standard deviation, variance and range were determined.

Results

Quality and Quantity control

The quality and quantity of the extracted RNA including miRNAs upon completing the extractions was analyzed, this was performed for both the spiked-in samples and the non-spiked samples. Spiked-in samples were spiked with miR-223 before the extraction. For the spiked-in samples 2µL of elute was used. As for the non-spiked samples, 4µL was used in order to inspect the quantity and obtain readings. Unfortunately, all of them were out of range, with the exception of two-spiked in samples that were 10^5 copies/µL.

However, for quality control all results were obtained for all samples. Raw data of the spiked-in and non-spiked samples collected with DS11+ Spectrophotometer (DeNovix) can be found in appendix (Appendix 1, Table I and II). Based on the raw data a mean ± standard deviation (SD) was determined for the 260/280 measure of both spiked-in and non-spiked samples (Table 1.) in order to determine which samples had better quality (purity). For RNA desired purity would be ~2.0 260/280 absorbance ratio.

Table 1. Mean value ± standard deviation (SD) of the 260/280 absorbance ratio of all the spiked-in (n=12) and non-spiked elutes (n=10).

Sample	Mean 260/280 ± SD
Spiked-in samples (n=12)	1.28 ± 0.43
Non-spiked samples (n=10)	1.50 ± 0.20

Two-Tailed RT-qPCR

Having performed a cDNA synthesis and later on RT-qPCR the results were obtained and analyzed. The amplification was sucessful for spiked-in elutions. Out of 12 eluted samples eight were

analyzed, as for the all non-spiked samples, all 10 elutions extracted were analyzed. In total four qPCRs were run and analyzed, and the most optimized standard curve was selected and used for absolute quantification of non-spiked elutions. Standard curve that was selected to be worked with was the standard curve of the third qPCR run. The negative controls of the run were –RT and NTC, and all negative controls remained undetected for this specific run.

RT-qPCR standard curve

A standard curve of a qPCR is an indication of the linearity, efficiency and reproducibility of the qPCR. In order to optimize the two-tailed RT-qPCR technology (Bio-Rad laboratories, 2006) a standard curve was made in a 10-fold serial dilution and analyzed. No exclusions were made in the serial dilution in order to optimize the standard curve, all points were included. The first serial dilution (SD1) was made and ran in the qPCR, and seeing how the efficiency and the R^2 values were not optimal another serial dilution was made (SD2). SD2 contained the same dilution range and resulted in good values, thus SD2 was the dilution used for all qPCR runs. As mentioned, standard curve deemed most optimized was the standard curve of the third qPCR run (Figure 1).

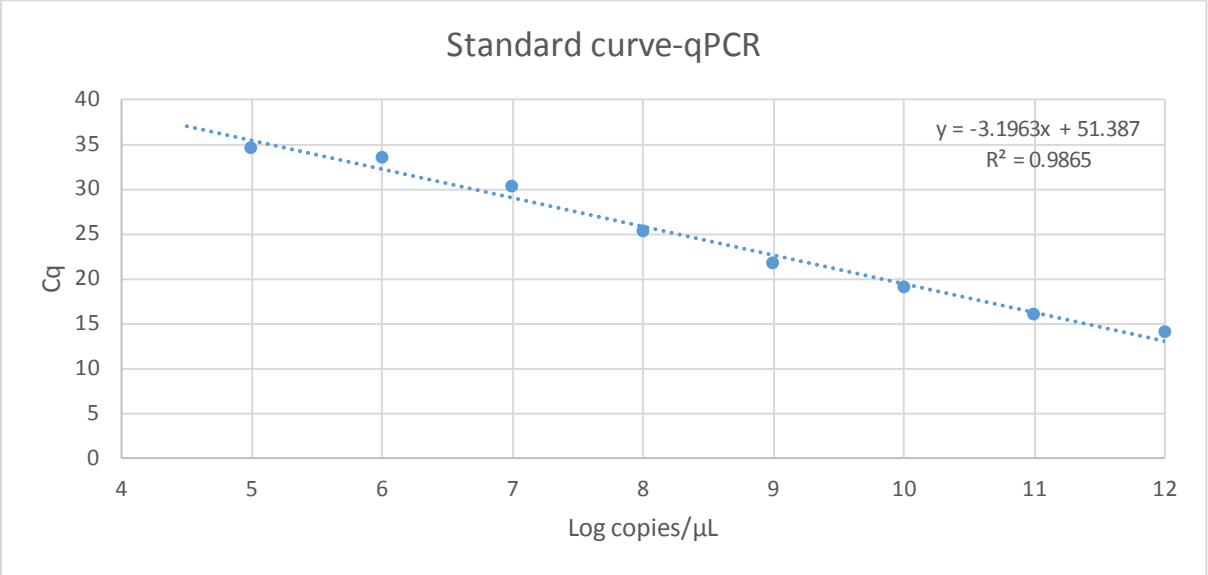

Figure 1. Standard curve of the third qPCR run, where Cq values (Appendix 2, Table III) are plotted against the logarithmic value of the copies/µL. R^2 value= 0.9865 and the slope (m) = -3.1963.

The curve was plotted in Excel, where a slope and R^2 value were obtained. The efficiency of the standard curve was 105% for the SD2 dilution of miR-223. Cq values and means of the SD2 can be found in the Appendices (Appendix 2, Table III).

A standard curve was constructed (Figure 1) and the recommended parameters for an optimized qPCR were evaluated (Table 2).

Table 2. Slope (m), efficiency and efficiency in percentage of the SD2 10-fold serial dilution of miR-223

	miR-223
Slope (m)	-3.1963
Efficiency (E)	E= 2.055
Efficiency in %	E= 105%

Melt Curve analysis of the Two-tailed RT-qPCR

SYBR® Green is a dye used for the quantification of the double-stranded DNA and it works by binding to DNA during qPCR. During heating the melting temperature (T_m) was reached and at that temperature DNA denatured and the fluorescence was reduced. A melt curve was constructed in qPCR and the results showed if the samples were contaminated, if the primers worked and if primer-dimers were formed. Primer sequences (RT and qPCR- forward/reverse) can be found in the Appendices (Appendix 3, Table IV). The melt curve that was selected to be analyzed was the melt curve of the standard curve that was deemed most optimized (SD2 of the third qPCR run). Along with the SD2, three non-spiked elutes (S4-S6) along with positive (E3-10^5) and negative (NTC and –RTs of the non-spiked elutes) controls were analyzed in the melt curve analysis. NTC of the qPCR run had no amplification; all other samples were amplified and formed a dissociation curve (Figure 2).

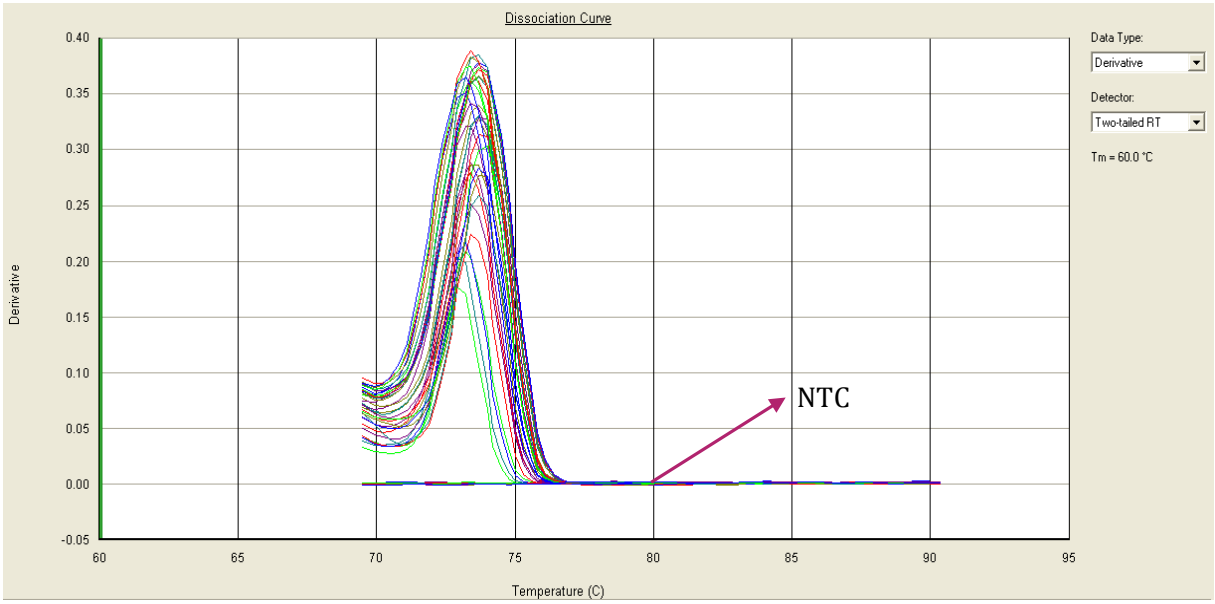

Figure 2. Melt curve analysis for a qPCR run of miR-223 at the mealting temperature (T_m) = 60°C. NTC as negative control- no amplification.

As seen on Figure 2, the results indicate a single uniform peak, without any background noise and other peaks.

Absolute quantification

Following the making of the standard curve, the non-spiked, i.e. unknown samples were plotted on the standard curve to obtain the quantity of the unknown elutes. Along with the quantity, the mean Cq and the standard deviation of the non-spiked samples were determined in SPSS (IBM).

After the successful quantification the results were visualized in Excel by plotting the now known quantities of the non-spiked samples onto the standard curve (Figure 3). Along with the non-spiked samples, SD of the Cq values was plotted onto Figure 3.

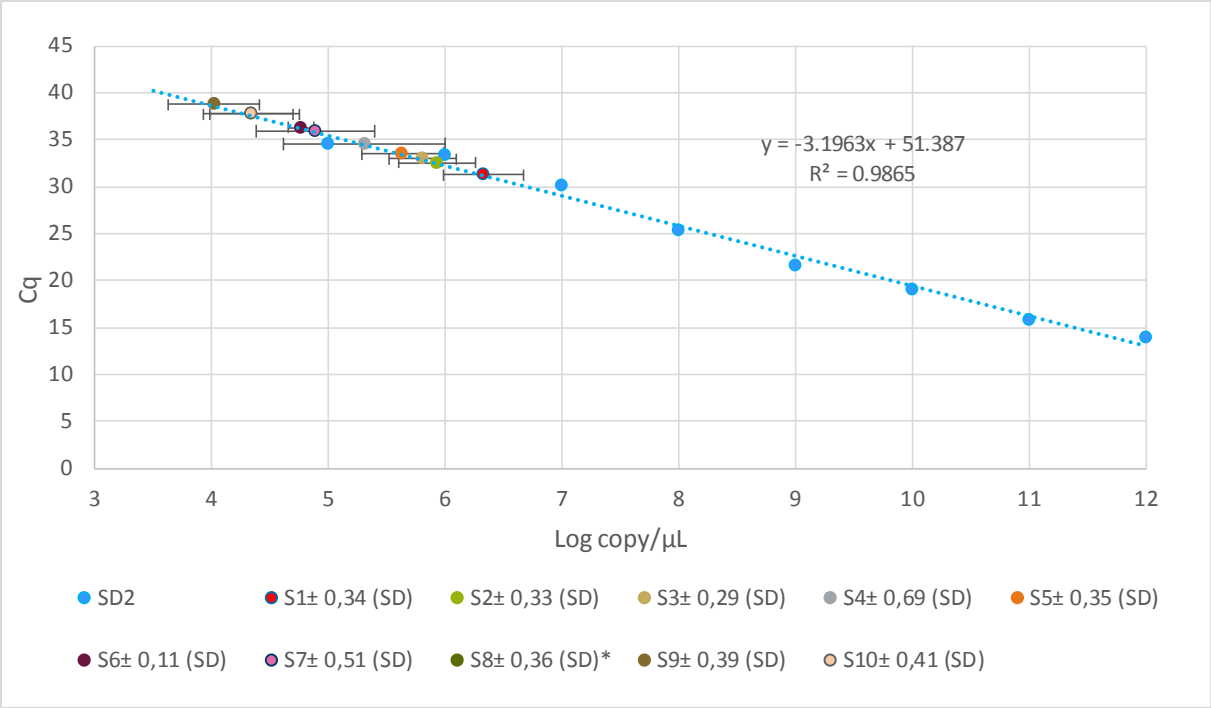

Figure 3. Non-spiked samples plotted onto the SD2 standard curve to visualize the quantified results. Blue dots represent the serial dilution ranging from 10^{12} to 10^{5}, while all the other colors represent the non-spiked elutes that were quantified (S1-S10). Along with the samples on the SD2 standard curve, SD of the Cq values of the non-spiked elutes was presented on the figure.
*S8 and S10 have similar values, so S8 value is not presented on the figure since the values lie in the same place. However, SD of the S8 and S10 samples have different values and are presented on the figure respectfully.

Horizontal error bars were selected on Figure 3 in order to better present the SD values. It was possible to better present the SD values on the horizontal error bars since the margin of difference on the x-axis is lesser than the margin of difference on the y-axis, so the results are more apparent.

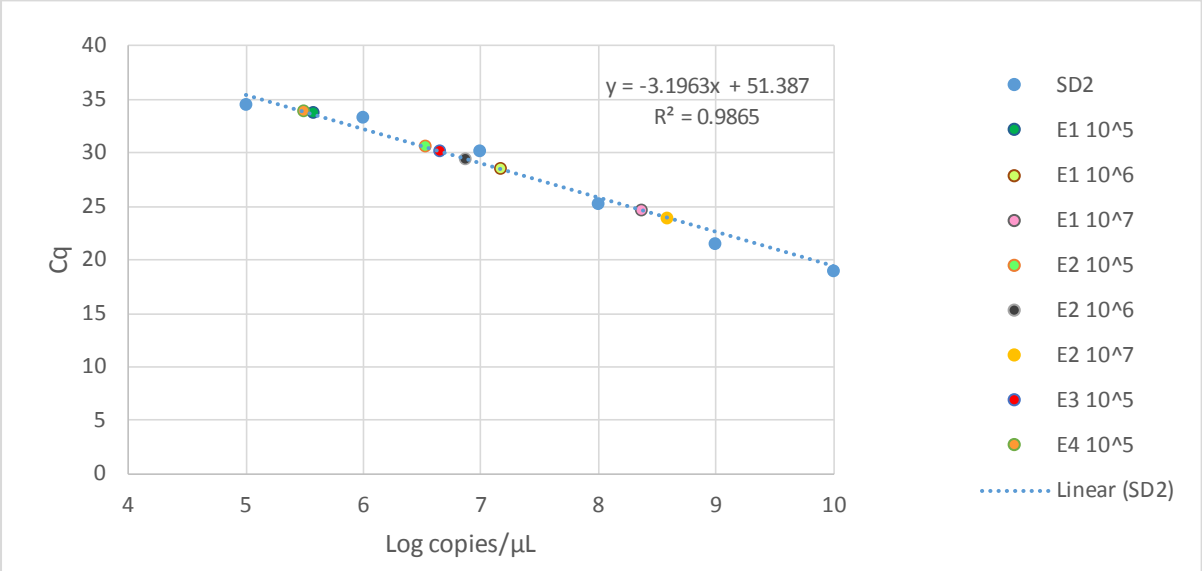

Figure 4. Spiked-in samples plotted onto SD2 serving as a positive control for the non-spiked samples. E= elution + which elution it is (1-4) + which copy number do the elutions have (10^{5}, 10^{6} or 10^{7}).

To better represent the result and where the non-spiked samples lie on the standard curve Figure 5 was created. The highest log concentration is S1=$10^{6.33}$, and the S9 has the lowest log concentration of S9=$10^{4.02}$. To remove the great deal of significant figures that are presented once the log copy numbers are converted into copy numbers, it was decided to have the log copy numbers remain as they are and not convert them.

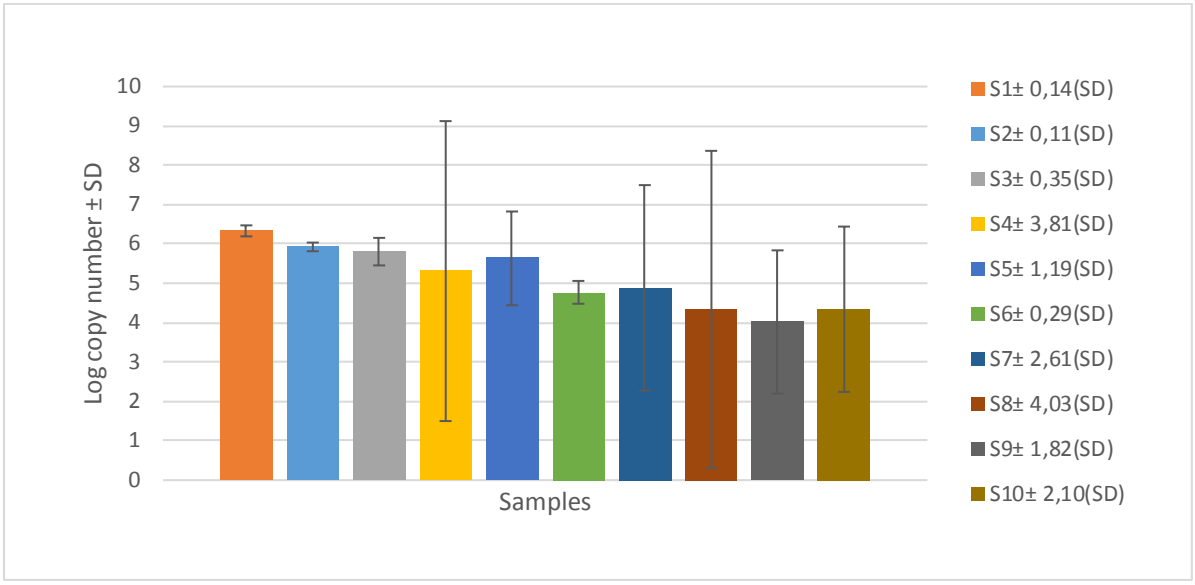

Figure 5. MiR-223 expression of the non-spiked samples (S1-S10). The logarithmic copy number of the non-spiked samples as well as their individual ±SDs are presented. Log copy numbers: S1=6.33, S2=5.93, S3=5.81, S4=5.31, S5=5.64, S6=4.77, S7=4.89, S8=4.34, S9=4.02 and S10=4.34.

Statistical analysis

Due to working with one miRNA- miR-223, one volume (100µL) and one method (manual work with the miRNeasy kit- Qiagen) no comparisons were performed for the statistical analysis. Only comparison done was the comparison of the quality of spiked-in and non-spiked elutes between their medians. Descriptive statistics of all elutes were performed along with the Excel analysis to obtain the standard curve and quantity of the extracted miRNAs. The figure depicting the descriptive statistics of all elutes (spiked-in and non-spiked) - mean, median, standard deviation, range and variation, can be found in the Appendices (Appendix 4, Table V and VI).

Discussion

The project sought to find out if it was possible to quantify the unknown concentrations of miR-223 extracted from plasma samples using the novel two-tailed RT-qPCR (TATAA Biocenter) technique and absolute quantification from a total volume of 100µL blood plasma. Previous publications (Androvic et al., 2019) and thesis work (von Ehr, 2020), worked on extracting miRNA from a total volume of 200µL blood plasma, however, the thesis workers have also worked with the total volume of 100µL blood plasma and compared the quantification to the volume of 200µL (von Ehr, 2020). These results indicated that a total volume of 100µL blood plasma might be sufficient for the extraction of miRNA, and thus this was tested using the manual miRNeasy Serum/Plasma Advanced Kit (Qiagen) and later quantified using the absolute quantification.

TATAA Biocenter developed the two-tailed RT-qPCR technique as a technique that would be specific and cost-effective in quantifying miRNAs (Androvic et al., 2017). Specifically structured primers for reverse transcription have an increased binding strength, which in turn leads to an increased sensitivity when quantifying (Androvic et al., 2017). This method and absolute

quantification were performed in order to successfully get a concentration of unknown elutes (non-spiked) extracted from human blood plasma (Iguchi et al., 2018). Absolute quantification is a technique of quantifying where it determines the detection from an absolute number of copies (Bissels et al., 2009). A standard curve is created with known concentrations and the samples with unknown concentrations are compared to the standard curve in order to determine their concentrations.

When it comes to the extraction of miRNAs, in general there has not been a standardized method and/or kit that would be used for miRNA extraction. On the market currently, there are plethora of kits that are being used for extraction (Brunet-Vega et al., 2015). Which one is used for a project depends on the budget, availability, time and equipment that is available to work with. Additionally, depending on which kit is used it might influence the yield and purity of miRNA (Wright et al., 2020). As stated, for this project miRNeasy Serum/Plasma Advanced Kit (Qiagen) was used, a phenol-free kit that can be worked with on the bench and has no need for a fume hood. The kit contains UCP columns that ensure pure RNA elutes with less contaminations. Additionally, smaller volumes of elutes are collected after the extraction thus giving more concentrated samples (Moret et al., 2013). An example of an influence on yield and amplification performance would be different RNA carriers or glycogen that have been shown to increase the yield or performance (Moret et al., 2013). Androvic at al. (2019) also implicated this finding in their study where they suggested that glycogen should be added to increase the robustness and efficiency of the miRNA isolation with the miRNeasy Serum/Plasma Advanced Kit (Qiagen). In a study performed by Wright et al., (2020), across the operational factors miRNeasy Serum/Plasma Advanced Kit (Qiagen) showed to be one of the top three performing kits.

Quality and quantity control (QC) were analyzed. Qubit microRNA Assay (Life Technologies) was used for quantity control and is an assay that grants clear and precise quantification of small-RNA including miRNA using the Qubit Fluorometer (Life Technologies). The assay is extremely selective for miRNA. Despite the reagent not being entirely selective for miRNA, it was possible to successfully quantify miRNA in pure samples at levels as low as 0.5 ng/µL according to Qubit MicroRNA Assay Manual (Thermofisher Scientific, 2015). As mentioned previously almost no values were collected for the quantity control performed with Qubit (Life Technologies), save for two readings for the spiked-in samples containing 10^5 copies.

The quantity results were not surprising since a challenge has been noticed in extracting miRNA from bodily fluids or in this case blood plasma (Cui and Cui, 2020; Blondal et al., 2013), however, some plausible reasons as to why the quantity results were not successfully obtained have been listed.

The possible reasons were that there was either no small-RNA (including miRNA) to quantify, the Qubit assay was not sensitive enough to detect the low levels of small-RNA (including miRNA) in the samples, the MasterMix was not properly made or the Qubit standards were not successfully set up (Garcia-Elias et al., 2017). Considering that, other thesis workers (Personal communication, April 29, 2021) had sucessful readings the standards should be okay. In addition, considering that the Qubit MasterMix was prepared multiple times and was occasionally shared between other thesis workers where they had readings it should be safe to assume that the standard and the MasterMix could be excluded as potential reasons as to why almost all of the quantity control measurements were out of range. The other two plausible reasons could be an explanation as to why the readings were out of range. However, seeing as the RT-qPCR quantification was successful for all samples the most probable reason as to why the measurements were out of range is that the Assay was not sensitive enough to pick up the low quantities of miRNA in the sample. As stated in the Qubit MicroRNA Assay Manual (Thermofisher Scientific, 2015) the range of detection is between 5ng/mL to 500 ng/mL as the core assay range and 0.05ng/µL to 100ng/µL for initial sample concentrations.

As for the quality control (Appendix 1, Table I and II), it was successfully preformed for all results using the DS11+ Spectrophotometer (DeNovix). An accepted measure for a pure RNA reading should be around ~2.0. Spectrophotometers measure the fluorescence at different absorbance wavelengths (Desjardins and Conklin, 2010). The wavelength most commonly used to measure the purity or quality of a nucleic acid or protein, in this case RNA, is 260/280 nanometer (nm) absorbance (Garcia-Elias et al., 2017). A lower value in this absorbance measurement for a sample could indicate a presence of proteins or other contaminants. A contaminant that is often seen in the extraction of miRNA is phenol, however miRNeasy Serum/Plasma Advanced Kit (Qiagen) contains no phenol and thus does not contaminate the purity of elutions in this way. Naturally, other contaminants may be present in the sample if it is handled carelessly. The ratio of 260/230 absorbance is another indication of the purity of the sample; this ratio may indicate the presence of organic contaminants such as carbohydrates and phenol.

Another thing to take into consideration is that if the yield concentration is low the 260/230 absorbance ratio will be low as well (El-Khoury et al., 2016). Appendix 1 Contains the raw data of the purity of both spiked-in and non-spike samples, additionally the mean values of the 260/280 absorbance ratio for the spiked-in and non-spiked samples (Table 1). Both in the appendix and in the table the gotten purity results are lower than the value considered ideal. The non-spiked samples present a better purity value- 260/280 absorbance ratio = 1.50. As there is no phenol present in this kit the values could have been contaminated in several other ways. There could have been an ethanol transfer when changing columns, it could have also been a matter of not accurate enough handling and inconsistent pipetting. Multiple causes could have been the reason why the purity values are lower than they should be. As mentioned before with 260/230 absorbance ratio it could have been the low yield value that lowered the measurements (El-Khoury et al., 2016).

Another factor that should be considered when talking about the expression level and yield of miRNA in blood serum/plasma is hemolysis (Kirschner et al., 2011). Hemolysis is rupturing of the red blood cells and their release in the surrounding fluid, e.g. blood plasma. An improper handling of the blood samples, such as their collection and processing, can cause *in vitro* hemolysis (Sowemimo-Coker, 2002). Improper and prolonged storage as well as excessive forces, such as violently shaking the tube, can be factors for hemolysis. This in turn has repercussions on the lab work, as it can cause inaccurate results by contaminating the surrounding plasma. Clinical labs demand QC for hemolysis (Androvic et al., 2019) when working with blood plasma, however, for this project no tests for hemolysis were performed, as it was not suspected that hemolysis was the reason for any irregularities in the results.

The blood plasma was spiked with a synthetic miR-223 miRNA and the samples were spiked with three different concentrations of the spike-in- 10^7, 10^6 and 10^5 copies. Spiked-in samples were serving as a positive control during the qPCR runs of the non-spiked samples, while no reverse transcription (-RT) and no template control (NTC) served as negative controls. In total four qPCR runs were performed out of those three of them contained non-spiked samples (three samples per plate, except the last one which had four). The run that did not contain any non-spiked samples was the first run that only contained spiked-in samples to test the method. All of the runs has successfully quantified samples. Only optimization performed was re-doing the serial dilution (SD2 used for the project) used to make the standard curve in order to obtain better efficiency and coefficient of determination (R^2) value (Figure 1).

The standard curve constructed in Excel by plotting the dilution of miR-223 was used to test the parameters (R^2 value and efficiency) and check if they have been optimized. An optimal R^2 value is ~0.98 and an optimal slope for the calculation of the efficiency is between -3.3 and -3.5, which gives 90-100% efficiency (Bustin et al., 2009). Good efficiency value falls between 90-110%

efficiency. The efficiency was calculated and the efficiency of the miR-223 SD2 standard curve was 105% while the R^2 value was- 0.9865 (R^2 value-Figure 1; Efficiency-Table 2), which fall in the range of the good efficiency and R^2 value (Bissels et al., 2009). Along with efficiency and R^2 analysis, a melt curve analysis was performed (Figure 2). The melt curve shows a single, uniform peak which indicated that there are probably no contaminations and background noise. A clear amplification was seen without primer-dimers indicating that the primers had a desired function in the amplification. Negative controls were undetected (no amplification) which indicated that there were no contaminations, e.g. in the wells or in the MasterMix (Bustin et al., 2009) as well as no formation of primer-dimers. However, this was not the case for all qPCR runs. In the last qPCR run, there was a detection in two out of three NTCs and two –RT wells in the later cycles indicating that some kind of contamination occurred. Because of the amplification in a few wells containing the negative controls as well as slightly worse efficiency and R^2 value, the last qPCR run was not selected as the best standard curve that was analyzed. The third qPCR run was selected for that (Figure 3).

Following the making of the standard curve, the non-spiked, i.e. unknown samples were plotted on the standard curve to obtain the quantity of the unknown samples. In order to determine the quantity of the miRNA from the extracted samples (non-spiked, i.e. unknowns samples) absolute quantification method was used (Boulter et al., 2016). By comparing, the unknown samples to the known concentration of the standard curve (SD2) the unknown sample were successfully quantified. As seen in the results in Figures 3. and 5. a variation in the copy number of non-spiked samples can be seen. The variation is not considerable but it is noticeable. This in turn could be a result of various reasons, such as variation of miRNA levels in the blood plasma as well as handling, storage conditions, and storage duration before processing (Iguchi et al., 2018). When it comes to the SD of the samples it was included separately for Cqs (Figure 3) and log copy numbers (Figure 5). The SDs were calculated for each sample individually and were presented on the graphs to visalize the approximate margins of error. As seen on the figures some samples have large margins of error and additional optimization on that part is required. For the sake of a more concise reading it was decided to leave the log copy numbers as they are and not convert them into copy numbers in order to reduce the number of significant figures. As previously stated there is a variation in both the miRNA levels in the blood plasma and SD of the log copy numbers (Figure 5), these variations are most likely to the handling of samples and the variation in the Cq values of the samples. In order to present more tangible results for possible future projects, some optimization is neccessary.

As for the spiked-in elutes that served as a positive control it was noticed that the concentrations of the synthetically spiked-in samples were slightly highert than expected (Figure 4). This in turn formed some questions about the accuracy of the synthetic spike-in. The spike-in elutes that had 10^5 copies and contained a Cq value around 33 gave the most accurate results, while the ones whose Cq values were around 30 had too high copy number and their concentrations on the Figure 4 were not as accurate. Some optimization regarding Cq values would have been preferable in order to get more accurate results of the positive control to be more secure in the results obtained from the non-spiked elutes. However, considering the occupancy of the qPCR during the thesis project this optimization was not possible.

Statistical analysis was planned initially; however, as no quantity measurements were obtained with Qubit (Life Technologies) no statistical analysis was done for this part (i.e. test of normality, parametric or non-parametric test). Seeing as work was done with one kit- miRNeasy Serum/Plasma Advanced Kit (Qiagen), for one volume of blood plasma- 100µL and with one method-manual, no comparisons could be performed here. Only statistical analysis performed

were descriptive statistic indicating mean, median, standard deviation, variance and range. Raw data of the descriptive statistic can be found in the Appendices (Appendix 4, Table V and VI).

Conclusion

The current research ongoing with studying miRNAs as biomarkers for sepsis as well as other diseases has a lot of potential (Sandquist and Wong, 2014). As more research on this topic is being conducted, more and more methods are being optimized to be more precise, cost less and give faster results (Mustafić et al., 2018). This is exactly what is needed to improve the response and reaction time to sepsis. It is somewhat difficult to diagnose sepsis since the symptoms resemble a variety of other diseases. Additionally sepsis develops very fast, so there is a possibility that it would not be noticed in time as septic shock occurs fast and leads to organ shut down and in the end death (Seymour and Rosengart, 2015). Currently c-reactive protein (CRP) and procalcitonin (PTC) are the most used biomarkers to recognize sepsis (Ljungström et al., 2017), however alternative biomarkers such as miRNAs are being examined as potential biomarkers for sepsis in hopes that their development will potentially bring novel methods that could help with developing treatments (Dumache et al., 2015). It has been seen that the mixture of pro- and anti-inflammatory biomarkers in a multimarker panel could aid in identifying patients who are starting to develop severe forms of sepsis before the sepsis progresses too much and organs shutdown (Seymour and Rosengart, 2015; Ljungström et al., 2017). Sepsis biomarkers have the capability to supply better and faster information than other techniques and thus help diagnose the condition earlier, observe the effectiveness of the treatment and change it in time if needed. The quantification of a lower volume of blood plasma was successful using the Advanced Qiagen kit and the two-tailed RT-qPCR, the next step could be to further the technique and work with multiple miRNAs and to try the method on the blood of septic patients collected by biobank. By furthering the technique even more it is possible to allow better response time to sepsis in clinical settings. Moreover, miRNAs are not limited only to sepsis research but can also be used as indicators of several other diseases (Ardekani and Naeini, 2010), prime example would be cancer (Peng and Croce, 2016). Novel research sees a lot of potential in using miRNAs as biomarkers for the detection of sepsis as well as other diseases and the upside of successful detection through miRNAs would be the cost effectiveness, response time and more disease-specific treatments. All this in turn has effect on society as well, as it would be desirable to have a standardized method in diagnosing diseases and thus reacting faster. More research is needed, however, the method tested in this project has potential in becoming a simple and quick novel tool in diagnosing sepsis in the early stages and thus saving lives.

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Appendices

Appendix 1- raw Spectrophotometer data of the samples

Table I. Spectrophotometer results of the non-spiked elutions (n=10) for the 260/280 and 260/230 absorbance ratios as well as total RNA quantity in ng/µL. S= elution + number which elution it is.

Sample	260/280	260/230	ng/µL
S1	1.434	0.046	0.784
S2	1.344	0.021	3.051
S3	1.715	0.045	3.993
S4	1.475	0.045	3.290
S5	1.455	0.013	1.963
S6	1.335	0.093	6.224
S7	1.332	0.060	1.037
S8	1.392	0.037	11.653
S9	1.511	0.031	3.579
S10	1.978	0.064	1.634

Table II. Spectrophotometer results of the spiked-in elutions (n=12) for the 260/280 and 260/230 absorbance ratios as well as total RNA quantity in ng/µL. Each copy/µL (10^5,10^6 and 10^7) have four elutions marked as E (elution) + number which elution it is.

Sample	260/280	260/230	ng/µL
E1-10^5	1.124	0.010	2.448
E2-10^5	1.180	0.245	7.577
E3-10^5	1.537	0.091	2.506
E4-10^5	0.673	0.025	1.328
E1-10^6	1.337	0.161	6.882
E2-10^6	0.722	0.014	1.977
E3-10^6	0.872	0.026	2.617
E4-10^6	1.115	0.022	4.342
E1-10^7	1.794	0.302	2.884
E2-10^7	2.148	0.065	2.164
E3-10^7	1.479	0.033	4.820
E4-10^7	1.377	0.209	13.444

Appendix 2- SD2 Cq values, means ± Standard deviation

Table III. SD2 serial dilution ran in the qPCR indicating the Cq of individual triplicates, their respective means and ± standard deviation. Brackets () in the table indicate removed outliers that had a Cq variance greater than 0.5.

Sample (copies/µL)	Triplicate 1	Triplicate 2	Triplicate 3	Mean ± St.Dev.
10^5	34.53	35.03	(36.87)	34.78 ± 0.35
10^6	32.81	33.63	(34.86)	33.22 ± 0.58
10^7	(31.19)	30.38	29.85	30.12 ± 0.37
10^8	25.36	24.94	25.20	25.17 ± 0.21
10^9	21.86	21.70	22.08	21.88 ± 0.19
10^{10}	18.45	18.65	19.01	18.70 ± 0.28
10^{11}	15.52	15.38	15.19	15.36 ± 0.17
10^{12}	13.86	13.81	13.82	13.83 ± 0.03

Appendix 3- miR-223 nucleotide sequence and primer sequences

Table IV. miR-223 nucleotide sequence and primer sequences of the two-tailed RT and qPCR (forward/reverse) for miR-223 miRNA.

Synthetic microRNA used - miR-223	
miRNA sequence	CGUGUAUUUGACAAGCUGAGUU
Two-tailed RT primer sequence	AAATACACGTGCTAGACTCTACACACTTACTATAAGTAAGTAAGTAACCTATTAGAACTCAGC
Forward qPCR primer sequence	AAATACACGTGCTAGACTCTA
Reverse qPCR primer sequence	CGGCCGTGTATTTGACAA

Appendix 4- Raw data of the descriptive statistics

Table V. Descriptive statistics in SPSS- mean, median, standard deviation, variance and range of the Cq values of the non-spiked samples (n=10).

Sample	**Cq-S1**	**Cq-S2**	**Cq-S3**	**Cq-S4**	**Cq-S5**	**Cq-S6**	**Cq-S7**	**Cq-S8**	**Cq-S9**	**Cq-S10**
N	3	3	3	2	2	2	3	2	3	2
Mean	31,2367	32,5567	32,8867	34,3550	33,4950	36,2950	35,9333	37,7150	38,7400	37,6900
Median	31,3300	32,5100	32,7600	34,3550	33,4950	36,2950	35,8100	37,7150	38,6200	37,6900
Std. Deviation	,33975	,33247	,29143	,68589	,34648	,10607	,50639	,36062	,39395	,41012
Variance	,115	,111	,085	,470	,120	,011	,256	,130	,155	,168
Range	,66	,66	,54	,97	,49	,15	,99	,51	,76	,58

Table VI. Descriptive statistics in SPSS- mean, median, standard deviation, variance and range of the Cq values of the spiked-in samples (n=8). E=elution (E1-E4) and copies/µL- 10^5, 10^6 and 10^7

Sample	**Cq-E1-10^5**	**Cq-E2-10^5**	**Cq-E3-10^5**	**Cq-E4-10^5**	**Cq-E1-10^6**	**Cq-E2-10^6**	**Cq-E1-10^7**	**Cq-E2-10^7**
N	3	3	3	2	3	2	3	2
Mean	33,7033	30,2133	30,0667	33,9550	28,5367	29,1500	24,6467	23,7250
Median	33,7200	30,4100	30,1800	33,9550	28,5500	29,1500	24,6800	23,7250
Std. Deviation	,05686	,67678	,24090	,00707	,15044	,07071	,30139	,16263
Variance	,003	,458	,058	,000	,023	,005	,091	,026
Range	,11	1,31	,44	,01	,30	,10	,60	,23