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Optimization of Cell-free Plasma RNA Extraction for Downstream Application

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ABSTRACT

The growing interest in biomedical studies has brought RNA from biofluids including plasma, as promising candidates for genetics profiling. The precision and reliability of an analysis in downstream application such as NanoString nCounter® MAX Analysis System (NanoString Technologies, Seattle, WA)) depend on the RNA quality, purity and level. In this project, NanoString nCounter® miRNA panel was chosen due to rapid identification and ability to profile approximately 800 miRNAs per run which requires total RNAs from plasma with a minimum concentration of 33.3 ng/µL with 260/280 and 260/230 ratios of ≥1.8 for optimal results. Unlike tissues and cells, circulating RNAs in plasma are cell-free and are present in small sizes. However, the abundance of proteins and inhibitors in the plasma as possible contaminants could diminish the effectiveness of molecular isolation techniques and pose challenges in RNA isolation and quantification. This could skew data collection and elucidation. Therefore, the main objective is to determine the optimized plasma RNA isolation protocol to overcome problems in RNA quality and purity with regards NanoString nCounter® MAX Analysis System requirement. Several optimization steps were performed, including the addition of one chloroform extraction step with extra washing steps instead of conducting only once following the actual protocol. After conducting these steps, the average 260/280 ratio falls between 1.7 to 1.8, slightly increased compared to the results before optimization which was around 1.4 to 1.6 since these steps of optimization help to remove excess impurities including phenol and salt. Furthermore, increasing the incubation time in certain steps, for instance, after sample homogenization with Qiazol, during

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95% ethanol precipitation and after RNase-free water addition have boosted the RNA recovery allowing RNA concentration of 15 ng/µL and above to be obtained. Hence, the optimized plasma RNA isolation protocol was determined since several issues related to plasma RNA concentration and purity were significantly improved by performing the additional steps in the protocol.

Keywords: *Optimization, plasma, RNA extraction, NanoString,* d*ownstream application*

INTRODUCTION

Biomarkers have become prevalent in fundamental and clinical research since their applications are widely accepted and applied for both disease diagnostics as well as relevant clinical outcome prediction. The rising in biomarkers applications for disease diagnostic and prediction have demanded for safe, efficient, and specific target biomarkers. Due to some benefits, biological fluids including plasma and serum are often used as targets for biomarker identification as they require less-invasive methods of specimen collection compared to biopsy techniques and have an abundance of biological molecules specific to a particular disease or target-gene for analysis [1]. One of the most common biomarkers used in biomarker identifications and genetic studies is microRNA (miRNA) which is defined as small, approximately 22 nucleotides, non-coding RNAs that are very stable in biofluids despite of its size as well as fragmented physical which acts as a vital molecule at post-transcriptional level, capable of modulating gene expression [1,2].

Scientific research has proposed miRNAs as potential candidate biomarkers since many of their alterations are closely related to disease development [2]. With the altered expression in miRNAs, scientists can detect infection from pathogens like bacteria, viruses or different types of diseases and their development, including cancers, diabetes, cardiovascular disorders, and Alzheimer's disease from various biological fluids [1]. Although miRNAs are beneficial for clinical research, there are some challenges to be considered especially for complex biological fluids like plasma and serum. Plasma and serum are the portions of blood that are known to be cell-free, where nucleic acid materials are usually present in a very low level compared to other samples like cells and tissues. This very minute amount of RNA is prone to risk and probability of a significant amount of loss during RNA isolation. Furthermore, most miRNAs within the samples are associated with either microparticles such as microvesicles, exosomes and apoptotic bodies or with RNA-binding proteins and lipoprotein complexes. The presence of these proteins and inhibitors will only exacerbate the problems in plasma molecular isolation and quantification, preventing reliable results from being achieved [1,2].

Obtaining reliable results during RNA quantification is crucial before proceeding with an analysis since the functionality of an analysis is highly dependent on both RNA quality and purity to yield accurate, reproducible, and relevant results. In the present study, NanoString nCounter® miRNA panel was chosen as the platform of interest for genetic analysis. This technology

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implemented an amplification-free procedure that directly identifies, profile and validates individual miRNA biomarkers efficiently by digitally detecting 800 miRNAs in a single run. In comparison with microarray or PCR-based technologies, NanoString holds some benefits in term of its sensitivity even for low levels of targets since NanoString makes effective use of its hybridization step that directly approaches target sequences without the need of amplification or reverse transcription that could introduce variability and bias in future data analysis [3]. This platform requires a total RNA from plasma with a minimum concentration of 33.3 ng/µL, together with 260/280 ratio and 260/230 ratio of 1.8. By considering the characteristics and issues related to plasma RNA, optimization needs to be done on the current protocol in order to prevent and minimize issues in RNA quality and purity before subjecting to NanoString nCounter® Analysis System.

The optimization was done on Qiagen miRNeasy Serum/Plasma (Qiagen, Hilden, Germany) protocol, a kit that is designed specifically for cell-free total RNA isolation by utilizing the use of phenol/guanidine-based lysis and silica-based spin column mechanism promoting easier elution and lower RNA loss during extraction. The protocol of this kit is almost similar to the common phenol-chloroform RNA isolation protocol involving Trizol as the lysis buffer, hence the term "standard protocol" used in this paper shall refer to the Qiagen miRNeasy Serum/Plasma (Qiagen, Hilden, Germany) protocol.

NanoString nCounter® miRNA Panel is compatible with different extraction protocols and kits ranging from microRNA-specific or total RNA isolation kit, as long as all of the samples to be analyzed are standardized and extracted using the same kit and protocol, since different RNA extraction kits yield different efficiencies. Hence, extracting them with the same kit and protocol would yield more consistent results with minimal bias in the analysis.

EXPERIMENTAL

Plasma Preparation

The plasma was prepared following the protocol given by Qiagen miRNeasy Serum/Plasma (Qiagen, Hilden, Germany) Handbook [4]. The preparation started with blood collection which was done at Sultan Ahmad Shah Medical Centre (SASMEC) upon consent from an individual subject with approval from IIUM Research Ethics Committee (IREC). A volume of 5 mL of blood was withdrawn from the subject and transferred into BD Vacutainer Blood Collection tubes containing K2 EDTA and subjected to blood processing within 1 hour after the collection. The tubes containing blood samples were centrifuged using a swinging bucket rotor at 4°C and 1900 × g for 10 minutes forming an upper layer containing plasma and a lower layer with red blood cells.

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The upper yellow layer was carefully transferred into a new centrifuge tube without touching the intermediate buffy coat layer that separates the plasma from red blood cells and platelets.

Then, the plasma was further separated from cellular nucleic acid by centrifuging with fixed-angle rotor at 16,000 × g and 4°C for 10 minutes. The centrifugation step resulted in the formation of cleared supernatant which was transferred again into a new tube and the pellet formed at the bottom of the tube was discarded. The plasma sample was subjected to storage at -80°C before being used & further processed. The entire extractions either in the standard protocol and optimization protocols were done using the same plasma sample derived from the same individual subject.

Plasma RNA Isolation (Standard)

Plasma RNA isolation was done by referring to the protocol given by Qiagen (Qiagen, Hilden, Germany) [4]. First, the prepared plasma from a similar subject stored at -80 °C was thawed before RNA isolation began. After the sample was no longer frozen, 1000 µL of Qiazol Lysis Reagent was added & mixed with 200 µL of the sample by pipetting up and down. Sample homogenization continued by incubating the sample at room temperature for 5 minutes before the addition of 200 µL chloroform. The sample with chloroform added was shaken for at least 15 seconds, left untouched for another 3 minutes and centrifuged for 15 minutes at 12,000 × g at 4°C. Phase separation occurred on the sample, forming an upper aqueous phase containing the RNA, interphase, and lower organic layer with pinkish colour.

The upper aqueous phase was carefully pipetted and transferred to a new tube. The extraction continued with RNA precipitation by adding 1.5 volumes of 95% ethanol and mixed by pipetting or inverting. From the tube, 700 µL of the sample was transferred into a RNeasy MinElute spin column in a 2 mL collection tube and centrifuged at 8000 × g for 15 seconds at room temperature. This step was repeated with the remainder of the sample; the supernatant was discarded after each centrifugation. After RNA precipitation, the sample was washed by adding 700 µL Buffer RWT into the column, centrifuged at 8000 × g for 15 seconds at room temperature and flow-through was discarded. The step was repeated using 500 µL Buffer RPE and 500 µL of 80% ethanol.

Then, the RNeasy MinElute spin column was placed in a new 2 mL collection tube and centrifuged at full speed for 15 minutes with the lid of the tube opened. The column was then placed into a new 1.5 mL collection tube and subjected to RNA solubilization by adding 14 µL RNase-free water exactly to the center of the spin column membrane with white surfaces. Elution was done by centrifuging the sample for 1 minute at full speed and the eluted RNA was either subjected to RNA quantification or stored at -80°C.

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Optimization on the Plasma RNA Isolation Protocol

The optimization was done based on the standard protocol of Qiagen miRNeasy Serum/Plasma (Qiagen, Hilden, Germany) kit by increasing certain incubation periods and adding several additional steps into the protocol (Table 1). The first optimization was made during sample homogenization, and after the addition of Qiazol Lysis Reagent, the sample was incubated for another 1 or 2 additional minutes. So, instead of incubating the sample for 5 minutes following the standard protocol, the sample was incubated for 6 to 7 minutes before proceeding with chloroform extraction step. Chloroform extraction step began after the addition of chloroform until phase separation. In the standard protocol, these steps need to be done once, but the optimization was made by adding another chloroform extraction step by simply repeating the exact steps as in the standard protocol.

The following optimization continued after the addition of 95% ethanol during RNA precipitation. In the standard protocol, there is no need for incubation during precipitation, but the step was improvised by referring to the modification made by Xie in 2013 [5] involving the extension of an incubation period of 10 minutes following the addition of 95% ethanol. Then, during RNA wash, twice additional washing steps using 75% ethanol were added into the protocol after washing the RNA with Buffer RWT, Buffer RPE and 80% ethanol. The optimization proceeded with air-drying for at least 1 minute to remove excess ethanol carryover before RNase-free water addition. These entire extractions steps were combined and regarded as 'Optimized' protocol.

RNA Quantification

Quantification of RNA was done following Desjardins & Conklin [6] by cleaning the upper and lower optical surfaces of the microvolume spectrophotometer sample retention system by pipetting 2 µL of clean deionized water onto the lower part of the optical surface. The level arms of NanoDrop™ 1000 Spectrophotometers (Thermo Scientific, USA) was closed to ensure that the upper pedestal comes in contact with the loaded deionized water. Then, the level arm was lifted again, and the optical surfaces were wiped off with a clean, dry and lint-free lab wipe. The NanoDrop software was opened, and the Nucleic Acid application was selected. A blank measurement was done by dispensing 2 µL of elution buffer onto the lower optical surface. The level arm was lowered, followed by the selection of "Blank" in the Nucleic Acid application. As the blank measurement was completed, both optical surfaces were cleaned with a lab wipe. The extracted RNA was dispensed by 2 µL volume onto the lower optical pedestal and the level arm was closed. As the "Measure" was selected in the software, it automatically calculated and quantified the RNA concentration and purity ratios. The spectral image was reviewed to assess sample quality. The NanoDrop optical surfaces were cleaned following the earlier cleaning method.

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Table 1: Summary of the standard protocol and the optimized protocol

Standard Protocol	Optimized Protocol
Qiazol Addition & Mix	Qiazol Addition & Mix
Incubate for 5 minutes	*Incubate for 6 to 7 minutes
Chloroform Addition & Centrifuge	Chloroform Addition & Centrifuge
	*Repeat chloroform extraction step again using the upper aqueous layer formed
Add 95% ethanol	Add 95% ethanol
Transfer precipitated sample into MinElute spin column & centrifuge	Transfer precipitated sample into MinElute spin column & centrifuge
Wash RNA with: ● Buffer RWT ● Buffer RPE ● 80% ethanol	Wash RNA with ● Buffer RWT ● Buffer RPE ● 80% ethanol ● *75% ethanol (twice)
Centrifuge the MinElute spin column (open tube)	Centrifuge the MinElute spin column (open tube)
	*Air-dry for 1 minute
RNase-free water addition	RNase-free water addition
	*Incubate for 10 minutes
Elution	Elution

*Optimization and extra steps added into the new protocol.

RESULTS AND DISCUSSION

RNA Concentration Comparison

To compare the differences and effects of the optimization on RNA quality and purity, plasma RNA extractions were initially conducted using the standard protocol followed by the optimized protocol (combination of all optimization steps involved). All the extractions were done using plasma samples obtained from the same individual for a total of six replicates. In other words, the extractions for both the standard and optimized protocol were repeated for six times on the plasma

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samples from the same source. Results of RNA quantification obtained were compared side-by-side, based on the RNA concentrations (Table 2) as well as RNA purity readings indicated by 260/280 and 260/230 ratios (Table 6). Table 2 shows the comparison of RNA concentrations between the standard and optimized protocol.

Table 2: RNA concentration after extracting RNAs from plasma of the same individual subject using both standard and optimized protocols

Replicate(s)	Standard (ng/µL)	Optimized (ng/µL)	Percentage Difference (%)
1	6.83	19.00	178.2
2	18.0	15.30	-15.0
3	7.26	98.60	1258.1
4	7.52	29.20	288.3
5	5.85	19.20	228.2
6	8.30	13.60	63.9
Mean	8.96	32.50	262.7

Most RNA concentration readings on the optimized are slightly higher than the standard protocol ($p = 0.07$).

Based on the table, there were some improvements in the RNA concentration obtained using the optimized protocol compared to the standard protocol; the RNA concentration readings obtained using the optimized protocol were slightly higher than the standard protocol, except in replicate 2. For the standard protocol, the results ranged from 5.85 ng/µL to 18.00 ng/µL across all six replicates. The RNA concentrations slightly increased after extractions were optimized, resulted in a minimum concentration of 13.60 ng/µL while the maximum concentration obtained was above the requirement needed for NanoString nCounter® MAX Analysis System which was 98.60 ng/µL. These improvements could have resulted from the elongation of the incubation period and some additional incubation steps added in the protocol that aid in recovering more RNA during extraction. Nevertheless, after conducting further analysis on the RNA concentrations throughout 6 replicates for both the standard and optimized protocol, the probability difference was only approaching the borderline of significance ($p = 0.07$).

As mentioned before, all the extractions for both the standard and optimized protocols were conducted using plasma samples obtained from the same individual. However, the percentage difference in some of the replicates was observed to be inconsistent, for instance, the percentage increment in the RNA concentration on replicate 3 after the optimization showed a huge gap (1258.1% increment) compared to the other five replicates which were below than 300% difference and there was also a 15% reduction in the concentration of RNA after extracting using

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the optimized protocol on replicate 2. The exact explanation behind these issues remains unclear but, possibly, there might be some inconsistencies within the samples in terms of the contaminating factors that might vary while handling the extraction on each replicate and unequal total RNA present could probably be one of the factors that contributed to difficulties in maintaining a more stable series of results and high RNA concentration after extraction though similar protocols was applied.

Hence, this could be one of the possible explanations to justify the reason why replicate 2 yielded the highest concentration (18.00 ng/µL) in the standard protocol extraction and one of the lowest (15.3 ng/µL) in the optimized method. To testify the extent of effectiveness of certain optimization steps within the optimized protocol made, such as the extension on the incubation time during sample homogenization with Qiazol and the samples incubation with 95% ethanol during RNA precipitation which are associated to the improvement of RNA concentration [5,7], each of these optimization steps were labelled as Optimization 1a and Optimization 1b respectively, undergone a test and compared to the results of RNA concentration obtained using standard protocol. Table 3 shows the designation of the steps.

Table 3: Designation of the optimization steps which are associated with RNA concentration

Optimization steps associated to RNA Concentration	Designation
Longer period of incubation during sample homogenization with Qiazol	Optimization 1a
Increased incubation period during RNA precipitation with 95% ethanol	Optimization 1b

Effects of Certain Optimization Steps on RNA Concentration

The aim of this test is to identify the degree of efficacy on plasma RNA concentration after increasing the period of incubation during sample homogenization with Qiazol (Optimization 1a) and during 95% ethanol RNA precipitation (Optimization 1b). The test was conducted by extracting RNA three times (triplicate) using the standard protocol, Optimization 1a and Optimization 1b. Similarly, all of the replicates and extractions were conducted using plasma samples from the same subject. The comparisons of the RNA concentrations results are depicted on Table 4 and Table 5.

From Table 4, RNA concentrations were observed to be improved in all replicates after Optimization 1a, resulting in 35.3% to 78.3% increase in concentration and the probability difference was also identified to be significant ($p = 0.03$). Likewise, the extractions with Optimization 1b (Table 5) also positively affected the RNA concentration, yielding 12.17 ng/µL to 18.20 ng/µL which were closed to the RNA concentrations obtained in Table 4 after Optimization 1a (10.00 ng/µL to 17.19 ng/µL). Majority of the percentages difference in

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Optimization 1b were slightly higher than the percentages difference in Optimization 1a. However, both Optimization 1a and Optimization 1b showed significant difference to the standard protocol with probability difference of p = 0.03 and p = 0.04, respectively.

Table 4: RNA concentrations obtained after extracting using standard protocol and the protocol with longer incubation period during Qiazol sample homogenization (Optimization 1a)

Replicate(s)	Standard (ng/µL)	Optimization 1a (ng/µL)	Percentage Difference (%)
1	8.56	14.30	67.5
2	9.64	17.19	78.3
3	7.39	10.00	35.3
Mean	8.53	13.80	61.8

The average of RNA concentration after increasing the incubation period during sample homogenization was 6.45 ng/µL higher than the average for standard protocol (p = 0.03).

Table 5: RNA concentrations obtained after extracting using standard protocol and the protocol with extended incubation period during RNA precipitation with 95% ethanol (Optimization 1b)

Replicate(s)	Standard (ng/µL)	Optimization 1b (ng/µL)	Percentage Difference (%)
1	8.56	18.20	112.6
2	9.64	13.40	39.3
3	7.39	12.17	64.7
Mean	8.53	14.59	71.2

Positive and significant impact on RNA concentrations was observed after incubating the sample with 95% ethanol during RNA precipitation (p = 0.04).

Generally, sample disruption and homogenization are a necessary early step in extracting RNA from the sample. Thorough homogenization of samples is an important and crucial step in RNA extraction that could help to avoid the loss as well as degradation of RNA. Proper homogenization method commonly contributes to extraction efficiency, quality, and integrity of RNA [8]. This statement is supported by Grinstein [7], who mentioned that RNA quality and concentration is highly dependent on the duration of homogenization. In addition, most RNAs within the plasma are associated and co-existed with different proteins especially exosomes [1]. Therefore, proper sample homogenization is crucial to isolate the RNA from proteins. These statements can support the evidence regarding the increased in efficacy of RNA extraction in Optimization 1a towards RNA concentration.

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Incubation during RNA precipitation after the addition of 95% ethanol in Optimization 1b aids in proper and optimum precipitation, which in turn increases the recovery of RNA. The efficiency of an RNA extraction is highly dependent on the incubation process and duration since they promote appropriate flocculation and precipitation of the RNA before centrifugation [9]. It is also important to ensure that the incubation of the sample was done at room temperature since incubating the sample at low temperature could diminish the efficiency of precipitation. There is a high possibility that low temperature would increase the viscosity of the samples, which in turn affects the movement of nucleic acid within the sample and hinders proper RNA precipitation [10]. The statement was further supported by Ban *et al.* [9], who mentioned that low temperature promotes the increment of dielectric constant within the alcohol and allows salts to co-precipitate within the sample as well, which later leads to poor precipitation of RNA.

Other than these 2 optimizations, there are several other steps which are not tested in this optimization but might aid in improving RNA concentration. One of them is by incubating the sample with RNase-free water at room temperature according to the manufacturer and based on Qiagen's Handbook prior to elution [4]. However, this optimization step was not tested due to limitation in the quantity of RNA extraction kit. This step could ensure a complete absorption of elution buffer in the column membrane and allows the RNA to solubilize with it, as long as it is conducted at room temperature since the optimum range of temperature for the columns to yield optimal performance and quality is between 15˚C to 25˚C. Clogging of columns may occur if experiments are performed beyond the optimal temperature range, which eventually will alter the effectiveness of the column's filtration and elution [11]. In addition, increased starting volume sample could also be another method of optimization that might help in obtaining desirable and higher RNA concentration. This method was done by Spornraft [12] by using a starting volume sample of 9 mL instead of 200 µL in accordance with the guideline and protocol given by Qiagen [4]. Again, no attempt was done on this method of optimization due to limited and insufficient amount of plasma sample available for optimization.

RNA Purity Comparison

RNA purity is another important aspect that needs to be focused for reliable results in analysis. Commonly, RNA quality can be identified and measured based on the 260/280 and the 260/230 ratio when assessing with optical density (OD) measurement like NanoDrop Spectrophotometer or Bioanalyzer [13]. The RNA is generally considered as pure if both ratios fall within 1.8 to 2.0, while any ratios beyond the given range would be deemed as a contaminated sample. Inaccurate and false estimation of the 260/280 are frequently associated with residual chemical contamination originated from the RNA extraction procedures, while poor 260/230 are commonly related to either salt or protein contamination [13]. Table 6 illustrates the comparison of RNA purity between the standard and the optimized protocol by focusing on both 260/280 and 260/230 ratios.

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Table 6: RNA Purity Comparison between the Standard and Optimized Protocol.

Replicate (s)	260/280		Percentage Difference (%)	260/230		Percentage Difference (%)
	Standard	Optimized		Standard	Optimized	
1	1.41	1.67	18.4	0.14	0.08	-42.9
2	1.43	1.82	27.3	0.19	0.05	-73.7
3	1.64	1.87	14.0	0.14	0.90	542.9
4	1.41	1.80	27.7	0.07	0.41	485.7
5	1.31	1.83	39.7	0.08	0.11	37.5
6	1.60	1.79	11.9	0.67	0.10	-85.1
Mean	1.47	1.80	22.4	0.21	0.28	33.3

Significant difference only observed on 260/280 ratios after the optimization ($p < 0.001$) while 260/230 ratios showed no significant difference ($p = 0.4$).

Based on Table 6, the results of 260/280 ratios for the standard protocol were below 1.64, which was not enough to indicate a favorable ratio for 260/280. However, the readings of 260/280 ratios significantly increased ($p < 0.001$) after performing some of the additional steps in the optimized protocol and fell between 1.67 to 1.87 with percentage difference ranging from 11.9% to 39.7%. Similar to RNA concentrations, the cause of this varied changes in percentage might be due to varied amount of contamination present within the samples while conducting the extractions. The majority of 260/280 ratios on the optimized protocol were within the optimal range for pure RNA.

However, 260/230 were observed to be unstable and inconsistent in both the standard and optimized extraction throughout all the six replicates leading to fluctuation in the percentages difference. The cause of this issue remains unclarified as the difference between both protocols was not even close to significant ($p = 0.4$). Again, 3 of the optimization steps associated with the improvement in purity of RNA namely, double chloroform extraction, additional RNA washing with 75% ethanol and air-drying were tested individually and compared side-by-side with the results obtained from standard protocol. The designation for each of the steps are shown in Table 7.

Table 7: Designation of the optimization steps related to RNA purity

Optimization steps associated to RNA Concentration	Designation
Additional chloroform extraction step	Optimization 2a
Additional 75% ethanol RNA wash	Optimization 2b
Air-drying for at least 1 minute	Optimization 2c

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Impact of Several Additional Steps toward RNA Purity

This test was conducted to determine the possible impact of some additional steps involved including additional chloroform extraction step (Optimization 2a), additional 75% ethanol RNA wash (Optimization 2b), and air-drying (Optimization 2c) to the RNA purity. Each of these steps were repeated for 3 replicates and compared with the results from standard protocol to give some insights on whether or not each of the steps affects the purity of RNA. The results of Optimization 2a, Optimization 2b and Optimization 2c are shown in Table 8, Table 9 and Table 10 respectively.

From Table 8, the optimization of the protocol through the addition of chloroform extraction step has proved a significant difference (p = 0.001) compared to the standard protocol specifically towards 260/280 ratios. The average of 260/280 ratio for Optimization 2a was 1.84 and fell within the desirable range (1.80 to 2.00). In addition, it was also higher than the average of 260/280 ratios obtained from the standard protocol which fell near 1.51, with 19.2% to 22.8% changes throughout all of the replicates. In comparison to these, the means of 260/280 ratios obtained after performing additional 75% ethanol during RNA wash in Optimization 2b (Table 9) and after performing air-drying in Optimization 2c (Table 10) were 1.75 and 1.70 respectively. Despite of the difference in the average among three of the optimizations, all the 260/280 results yielded in the optimizations showed significant difference with the standard protocol.

Each of the additional steps related to improved RNA purity and performed in the optimization has different benefits and functions. For examples, the extra chloroform extraction step in Optimization 2a was performed following Toni *et al.* [14] which has aided in further removing excess contaminants, including salt and phenol from Qiazol, and helped in separating the RNA from lipid, protein, and DNA, which commonly present at the bottom organic phase after phase separation. However, this step needs to be performed carefully, especially when pipetting the aqueous layer from the sample to avoid accidental pipetting of the intermediate layer or organic phase containing lipids and proteins. Improper pipetting technique may draw these unwanted layers which consequently lead to undesired reading in purity.

Next, the addition of extra washing steps using 75% ethanol (Optimization 2b) was also referred to the optimization explained by Toni et al. [14] that helps in improving the 260/280 ratio by removing excess contaminants, especially any residual and excess salt carryover from the RNA extraction. Washing the RNA with 75% ethanol promotes solubilization of the salt allowing the salt to be eliminated from the sample. This step was performed by many researchers, including Junttila et al. [15] who successfully obtained desired values for both 260/280 and 260/230 ratios, and Roy et al. [16], whose 260/230 results were significantly improved after performing the step. Next, air-drying before the addition of RNase-free water was performed to remove excess ethanol carryover via evaporation. RNA extraction involving ethanol needs a lesser air-drying period compared to isopropanol since the volatility of ethanol is higher than isopropanol, indicating higher evaporation rate [17].

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Table 8: RNA Purity before (Standard) and after conducting additional chloroform extraction step (Optimization 2a)

Replicate (s)	260/280		Percentage Difference (%)	260/230		Percentage Difference (%)
	Standard	Opt. 2a		Standard	Opt. 2a	
1	1.49	1.83	22.8	0.08	0.33	312.5
2	1.54	1.89	22.7	0.12	0.15	25.0
3	1.51	1.80	19.2	0.88	0.20	-77.3
Mean	1.51	1.84	21.9	0.36	0.23	-36.1

Opt. 2a in the table is referring to Optimization 2a which showed a significant difference in 260/280 ratios (p = 0.001) but no significant difference shown in 260/230 between both (p = 0.3).

Table 9: RNA Purity before (Standard) and after conducting additional 75% ethanol washing on RNA (Optimization 2b)

Replicate (s)	260/280		Percentage Difference (%)	260/230		Percentage Difference (%)
	Standard	Opt. 2b		Standard	Opt. 2b	
1	1.49	1.74	16.8	0.08	0.30	275.0
2	1.54	1.73	12.3	0.12	0.17	41.7
3	1.51	1.77	17.2	0.88	0.95	7.95
Mean	1.51	1.75	15.9	0.36	0.47	30.6

Opt. 2b in the table is referring to Optimization 2b, had a significant difference with the standard protocol of RNA extraction (p = 0.004), however, 260/230 ratios for both does not show any significant improvement (p = 0.08).

Table 10: RNA Purity before (Standard) and after conducting air-drying prior to elution (Optimization 2c)

Replicate (s)	260/280		Percentage Difference (%)	260/230		Percentage Difference (%)
	Standard	Opt. 2c		Standard	Opt. 2c	
1	1.49	1.71	14.8	0.08	0.83	937.5
2	1.54	1.73	12.3	0.12	0.99	725.0
3	1.51	1.68	11.3	0.88	0.48	-45.5
Mean	1.51	1.70	12.6	0.36	0.77	113.9

Opt. 2c in the table is referring to Optimization 2c, and the ratio for 260/280 experienced a significant difference (p = 0.002) in contrast to insignificant difference in 260/230 between the 2 protocols. (p = 0.2).

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It is recommended to perform air-drying not more than 10 minutes since excess air-drying could make the RNA pellets becomes too dry and problematic during resuspension as well as elution. The optimization has slightly propelled the 260/280 ratio to the desired value for NanoString nCounter® MAX Analysis System. However, regardless of the thorough and carefully performed optimization steps, there was no improvement observed on the 260/230 ratio. Poor 260/230 reading is commonly associated with salt or protein contamination, but performing the steps mentioned, including chloroform extraction steps, unfortunately had no impact on the ratio despite the protein denaturing properties of chloroform [18] and solubilization of salt during RNA washing steps.

Plasma RNA Constitutes Problems for Proper Quality Control (QC)

Based on the handbook provided by NanoString [19], RNA extracted from plasma is expected to present at a very low level, which is commonly below the threshold amounts needed for reliable and optimum characterization from standard RNA quality check and quantifications, including those that involve absorbance measurements such as NanoDrop Spectrophotometer and Agilent Bioanalyzer. Signifying that having NanoDrop spectrophotometer alone as an instrument to determine the quality and purity of RNA is insufficient to obtain accurate results especially on 260/230 even if the extraction and quantification are done repeatedly [19]. Thus, a proper quality check is difficult to be performed to ensure that the samples meet the guidelines. This could be one of the reasons for the inconsistency of the 260/230 ratios obtained throughout the trials.

There are many methods to quantify RNA namely Nanodrop, Agilent Bioanalyzer as well as Fluorometric method using Qubit [1,20]. The choice of the quantification method is crucial since it is one of the aspects needed to be focused prior to analysis. Spectrophotometric methods such as NanoDrop and Agilent Bioanalyzer involving the application of UV-visible light at certain wavelengths to quantify the target of interest are traditionally known for their capability to measure the concentration of different molecules, including total RNA [1]. Nevertheless, these instruments are insufficient and ineffective enough for low concentrations samples such as cell-free plasma miRNA as the results often erroneous and inaccurate [20]. Different quantification methods yield different efficiencies and results. In 2013, Deben et al. [21] conducted an experiment and identified that fluorometric analysis is more appropriate and suitable to quantify RNA than spectrophotometry methods.

However, the RNAs quantified in the experiment were originated from tissue samples making the statement non-applicable to plasma samples since there is a large gap between the level of RNA in tissue and plasma samples [20]. Commonly, the estimation of the total yield of miRNA from plasma compared to the total yield of miRNA from tissue are around 1 to 10 ng and 1000 ng respectively [22]. Thus, the amount of cell-free miRNA in plasma is unreliable to be measured by either UV-absorption method such as Nanodrop and Agilent Bioanalyzer or even Qubit

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fluorometric quantification [23]. Other than these platforms and instruments, real-time PCR is also known to successfully applied and commonly used method to quantify circulating and cell-free miRNAs, which might suit for plasma miRNA [24].

The properties of miRNA within plasma samples contribute to some limitations in obtaining reliable and proper RNA purity and quality. Although both RNA quality and purity obtained were insufficient, but inaccurate quantification results from NanoDrop do not necessarily mean NanoString nCounter® MAX Analysis System will be drastically affected. The Nanostring nCounter® MAX Analysis System is sensitive and more advanced than the absorbance measurements involving NanoDrop, where individual molecules can be detected even, they exist at a very low level within a particular sample. Other than that, purity and the presence of contamination can also be detected via NanoString; for instance, this technology involves probes that are specific towards five mRNA house-keeping genes that are highly associated with cellular contamination. High cellular contamination is indicated by the increment in the counts for the 5 genes [19].

Limitations in Handling Plasma RNA

There are several limitations and considerations that need to be focused on prior to and while handling plasma RNA samples. One of the aspects that needs to be considered is during blood collection and process. It is possible that plasma and the RNA contents within the biological fluid can produce reproducible and robust results in different downstream application including NanoString nCounter® miRNA as long as the blood prior to plasma preparation are collected into tubes containing anticoagulant, either EDTA or citrate tube [19]. In contrast to other anticoagulants and additives such as Heparin and Sodium Fluoride which might obstruct downstream ligation or enzymatic steps regardless of whether some of them may slightly improve yield of certain miRNAs [25]. Besides, lysis of red blood cells (hemolysis) within blood samples can also significantly influence the total miRNA content and has become one of the common limitations to the abundance of miRNAs [19,26].

Hemolyzed plasma samples is indicated by their red or pinkish color and this condition of sample represents as a potential source of error related with the pre-analytical phase and usually resulted from improper blood collection and poor handling of sample for instance, inappropriate tubes mixing and careless related to transport and storage of the samples [19,27]. Hence, these mistakes related to hemolysis should be avoided for better and reliable future data interpretation. In addition to that, lysis of cells within the samples may also contribute to cellular miRNA contamination if the blood is not promptly processed once they are being collected [19]. Cellular miRNAs contamination within plasma sample might possibly influence circulating miRNAs [28] and consequently confound downstream data analysis and affect the quality of the results [29]. The

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sensitivity of RNA towards few factors such as Ribonuclease (RNase) and temperature also pose challenges in molecular research.

Although RNases are known to be important mediators in certain reactions of RNA metabolism, however, these ubiquitous enzymes are considered as nuisance contaminants due to their degradative characteristics towards RNA [30]. Hence, proper molecular techniques and good laboratory practices such as wearing proper attire and gloves as well as disinfecting workspace with 75% ethanol prior to plasma preparation and RNA extraction need to be applied. Furthermore, RNA storage is another aspect that should not be neglected as it is crucial to store RNA at -20˚C or -80˚C to prevent degradation [31].

CONCLUSION

After conducting the optimization on Qiagen miRNeasy Serum/Plasma protocol, several issues related to plasma RNA concentration and purity were significantly improved, although the requirements for NanoString nCounter® miRNA panel were not completely achieved. Considering the problems poses on plasma RNA extraction and the demand for plasma RNA as the target in biomedical studies, research on the optimization of plasma RNA extraction protocols are important and beneficial in providing some insights as well as ideas on which part can be optimized and improvised. There are some research focusing on the optimization of plasma RNA extraction, including Spornfart et al. [12], involving the optimization of plasma RNA, which utilized glycogen as the carrier molecule to enhance RNA recovery, likewise, the optimization made by Ban et al. [9] using glycogen, yeast tRNA and the combination of both while increasing certain incubation periods upon centrifugation. In the present study, the optimization involved a convenient, less complicated, and inexpensive method by utilizing reagents involved in the standard protocol. Hence, it would be suitable and useful, especially for a condition where resources are limited.

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CONFLICT OF INTEREST STATEMENT

The authors agree that this research was conducted in the absence of any self-benefits, commercial or financial conflicts and declare absence of conflicting interests with the funders.

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