A HOMEMADE EXTRACTION METHOD FOR SMALL RNAS FROM PLASMA SAMPLE

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SUMMARY

In recent years, the utility of miRNAs has proposed a dominant method to pre-diagnose and treat cancers. Although a variety of commercial kits for the extraction of RNAs are now available, they are cost-intensive, which does limit the studies of small-scale laboratories. Some others suggested different techniques like a Trizol-based method, which applies LiCl in the final precipitation step. However, previous studies proposed that LiCl cannot effectively precipitate small RNAs in contrast to $MgCl_2$. This project aims to modify and thus develop a more efficient homemade method for circulating small RNAs. After choosing the most suitable concentration of $MgCl_2$, the comparison between using $MgCl_2$, LiCl, and isolation kit (PureLink RNA Mini Kit) was also conducted. The methods were validated through testing the quantity and quality of recovered RNAs using concentration measurement, and the A260/A280 absorbance ratio. The primary result suggested that the final precipitation of small RNAs using the mixture of 0.5M $MgCl_2$ and ethanol provided the highest yield and quality of small RNAs, five times and ten times higher than LiCl and the isolation kit respectively. Additionally, the comparison between the developed method with the PureLink RNA Mini Kit showed that it exhibited a similar high purity range as the kit. As a result, it is promising that this method will be an effectively alternate way to extract small RNAs, which could reduce the dependence on using isolation kits.

Keywords: miRNA, plasma samples, $MgCl_2$, LiCl, isolation kit.

INTRODUCTION

MicroRNAs (miRNAs) are short, non-coding, and single-stranded RNA sequences (usually 19 - 23 nucleotides) that are responsible for post-transcriptional regulation of gene expression by binding to the 3′ untranslated region of certain mRNAs, and consequently, lead to mRNA degradation or translational inhibition. They serve as vital regulators of enormous biological processes, including cellular proliferation, differentiation, apoptosis, functional regulation of the immune system, and glucose, cholesterol, and iron homeostasis (Ma *et al*., 2013; Bartel *et al*., 2004). It is estimated that miRNA only account for 1 - 5% of the human genome, yet regulate more than 30% of protein-coding genes (Rajewsky, 2006). As discussed, miRNAs play an important role in the normal development of animals; thus, abnormal expression of miRNAs is a potential cue of many human diseases, including cancer. Most examined tumors have shown these unusual miRNA profiles compared to healthy people, which suggests that miRNAs could be used as biomarkers for cancer diagnosis, prognosis, and therapy (Rice at al., 2015). Over the past few years, a special type of miRNA, cell-free circulating miRNAs detected in the peripheral blood circulation and other body fluids, are reported to reflect the homeostatic status of the organism, and signs of disease progression as well. Beyond this, circulating miRNAs are really stable and resistant to endogenous RNase activity, which fosters their role as potential markers for disease diagnostics, especially cancer (Hamam *et al*., 2017).

Up to now, there are various methods to detect the expression of miRNAs such as northern blotting, bead-based flow-cytometry, microarray technology, and especially RT-qPCR. Regardless of the efficiency of these techniques, there is a need for miRNA to be effectively extracted from plasma samples as poor isolated miRNA samples woud affect the downstream analysis and lead to skewed results. Although there are a wide variety of commercial kits available for the isolation of miRNAs; their exorbitant costs limit the miRNA studies, especially small-scaled laboratories. Consequently, the development of the alternative protocols for the isolation of miRNAs specific for plasma samples is necessary.

Zununi *et al*. (2016) had developed another Trizol-based isolation method for miRNA with 2-step precipitation using potassium acetate and lithium chloride respectively. However, it should be noted that some small RNAs are not efficiently precipitated by lithium chloride and the transcript length of RNA for LiCl precipitation is required at least 300 nucleotides. Mg^{2+}, on top of that, is known to stabilize RNA-RNA interaction. $MgCl_2$ treatment significantly improved the precipitation of miR-141, whereas other salts such as NaOAc or NaCl did not show any effect (Kim *et al*., 2012). Thus, it indicates that the addition of $MgCl_2$ may be a useful way of minimizing bias and extracting all the miRNA species more evenly. RNAs are often crystallized from solutions of much higher Mg^{2+} ion

concentrations, up to 500 mM (Masquida *et al*., 1999). Another study suggested that the addition of magnesium chloride to a final concentration of 0.01 M and of 1 volume of ethanol results in the complete precipitation of polyribonucleotides having chain lengths of 7 to 200 or more (Razzell *et al.*, 1963).

In this project, based on the protocol established by Zununi *et al* (2016), the efficiency of $MgCl_2$ as an alternative salt for LiCl will be examined and from, that an efficient homemade technique for circulating small RNA isolation would be established, which could isolate them in a high quality and quantity compared to the isolation kit.

MATERIALS AND METHODS

Samples collection

Human whole blood from peripheral circulation was obtained from healthy volunteer individuals, who are in the age range of 18-30. The consent forms approved by the Hospital ethic group (Appendix) were provided to all volunteers in order to confirm their agreement and awareness before taking part in the research. The fresh blood samples were stored in the 2mL EDTA tubes to avoid coagulation and processed immediately after collection.

Plasma extraction

A volume of 2ml whole blood was centrifuged at 3,000 rpm for 10 minutes. Next, plasma was carefully transferred into a new 1.5ml eppendoft without touching the leukocyte layer, discard the samples if hemolysis occurs. The plasma samples were then centrifuged at 16,000 xg and 4^oC for 10 minutes. The supernatant was carefully transferred to a new tube without disturbing the pellet. Plasma was aliquoted in 1.5 mL RNase-free tubes and froze at −80°C immediately for future use.

Total RNA isolation

To denature proteins components, a volume of Trizol reagent was added to the collected plasma samples, mixed well and incubated at room temperature for 10 minutes. Next, chloroform (0.2 v/v) was added; by inverting the tubes vertically for 10 seconds, it was mixed and then separated aqueous and organic layers were created. Five-minute incubation at room temperature was applied to ensure that nucleoprotein complexes were completely dissociated. The total RNA in the aqueous phase was collected after centrifugation at 12,000 ×g for 12 min at 4°C.

Large RNA precipitation

Potassium acetate 3 M (1/10 v/v) was added to the collected supernatant from the previous step before incubating in -20 for 30 minutes. The samples were then centrifuged at 12,000 ×g for 12 min at 4°C. The pellet was discarded and the supernatant was transferred into a new tube.

Small RNA precipitation

Equal volumes of 2.5 M LiCl (v/v), 0.5 M $MgCl_2$ (v/v), 0.1 M $MgCl_2$ (v/v), or 0.04 M $MgCl_2$ (v/v) and 2 volumes of precooled absolute ethanol were added the to the samples. After that, the samples were incubated at -80°C for 2 h before going to the centrifugation at high speed 16,000 ×g, 4°C for 20 min. The pellets were dried and dissolved in 20 µL DEPC water, which was pre-incubated at 65°C.

Commercial isolation kit usage

The plasma samples were first treated by Lysis Buffer to disrupt and lyse the remained cells, then centrifuged at 12,000 x g for 2 minutes at 4^oC. After transferring the supernatant to a new tube, absolute ethanol was added to precipitate total RNA. Other cell components were filtered through the Spin Cartridge, followed by several washing steps by using Wash Buffer. The Spin Cartridge was next centrifuged at 12,000 xg for 1 minute at 4^oC RNA before eluting RNA in RNase-Free Water and re-suspended in the Recovery Tube.

Quantity and quality assessment of the extracted RNAs

The RNA concentration, and purity were confirmed using the relative absorbance ratio at A260/280 on a spectrophotometer (NanoDrop Technologies, Wilmington, DE, USA).

RESULTS AND DISCUSSION

0.5M $MgCl_2$ was the most suitable concentration for small RNA precipitation

In the step of determining the best $MgCl_2$ concentration, three different ones were compared in terms of RNA quantity and purity, including 0.5 M $MgCl_2$, 0.1 M $MgCl_2$, and 0.04 M $MgCl_2$. When the analysis of variance (ANOVA) was carried out, it suggested that the efficiency in the extraction of small RNAs was significantly different for 3 concentrations of $MgCl_2$ employed (Table 1). The average concentration of RNA extracted using 0.5M $MgCl_2$ is 116.8 ng/µL, compared to 53.5 ng/µL of 0.01M $MgCl_2$ and 38.3 ng/µL of 0.04 M $MgCl_2$. The A260/280 ratios of all three concentrations exceeded 1.70, indicating minimal protein contamination (Figure 1).

Table 1. ANOVA analysis of RNA yields at different $MgCl_2$ concentrations

Source of Variation	d.f.	F	p-value	F-crit
Between groups	3	7.01679	0.00317	3.23887
Within groups	16			
Total	19			

From the table of one-way ANOVA, it is shown that p-value (0.00317) is much smaller than 0.05 and the statistic F value is larger than the critical one, which means the null hypothesis of equal mean between 3 different $MgCl_2$ concentrations was rejected. Thus, they had significantly different effects on the RNA yield.

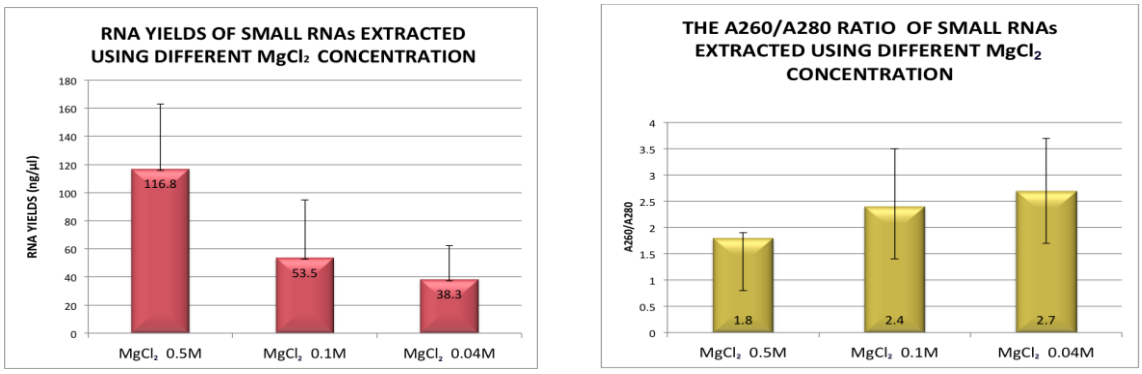

Figure 1. Comparison in the quantity and purity of RNA extracted between the use of different $MgCl_2$ concentrations

With a much higher average RNA yield and approximately high purity ratio, it was clear that $MgCl_2$ 0.5M showed the strongest potential in precipitating small RNAs.

$MgCl_2$ was more advantageous than LiCl and commercial kit

The efficiency of small RNA precipitation was then compared between 0.5 M $MgCl_2$, 2.5 M LiCl, and isolation kits. In terms of LiCl, its RNA yield was 25.7 ng/µL, which was substantially lower than those of 0.5 M $MgCl_2$. The A260/A280 absorbance ratio of RNAs extracted using LiCl was around the similar range of $MgCl_2$, with 2.5. When it comes to using the commercial isolation kit, the outcome showed that kit had a high purity as 0.5 M $MgCl_2$, yet, its RNA yields (12.2 ng/µL) was considerably lower than the new method developed (Figure 2). Based on this result, applying $MgCl_2$ at the concentration of 0.5M in the final precipitation of small RNAs was more advantageous than other RNA isolation methods.

Furthermore, the method established by Zununi *et al*. (2016) aimed to be used for various clinical samples, including plasma ones. Thus, it included the cell lysis step by applying lysis buffer such as Reporter Lysis Buffer (RLB), which can distort the purpose of isolating circulating small RNAs as we proposed. The protocol, then, was modified in the step of plasma extraction to ensure the collection of the proper profile of circulation RNAs.

From this, we conducted the detailed protocol with four main steps including 2-timed precipitation (Figure 3).

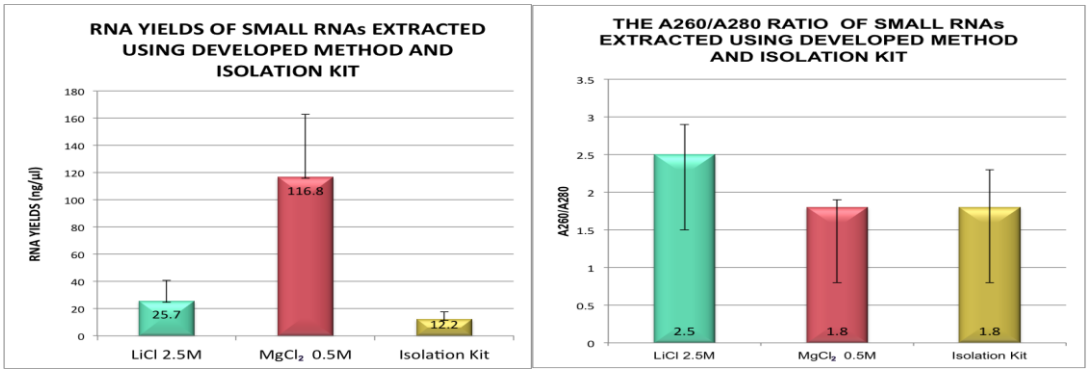

Figure 2. Comparison in the quantity and purity of RNA extracted between the different methods and isolation kit

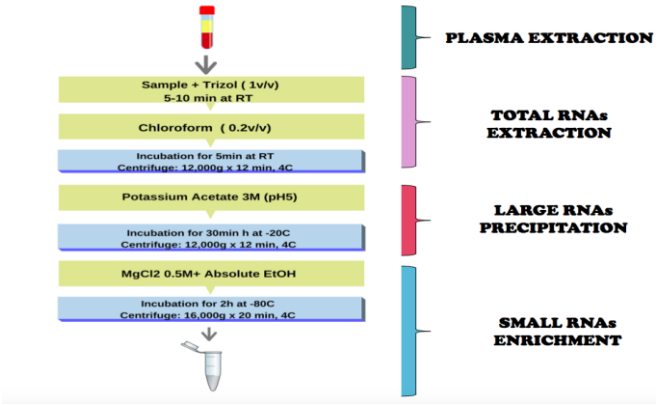

Figure 3. The detailed protocol

It is noted that Kim *et al*. (2012) had also investigated the effect of employing $MgCl_2$ to extract miRNA, yet, their protocol applied only one-step precipitation. This method could lead to the contamination of other large RNAs, which could affect further downstream analysis of qPCR. Because the large pool of RNA requires the primer designed for RT-qPCR to be extremely specific to ensure the amplification of the target miRNA. However, with the length of only 19-23 nt, miRNA amplification is much prone to error, and designing a highly specific primer is so perplexing. In this manner, using two-step precipitation to eliminate most large RNA as in our newly developed method is proposed to be the promising solution for this issue.

The stability of the newly developed method

The stability of the developed method was next examined in ten replicates with regards to the concentration and purity of extracted RNA. To minimize the biases because of different RNA profiles between different people and time, plasma samples were all obtained from the same healthy individuals in one period of time. The RNA samples isolated were considered significantly pure with the A260/A280 ration is 1.986±0.139, showing a stable pattern of samples' purity. However, the concentrations of extracted RNA were not steadily obtained, with the mean of 47.230±25.061 ng/µL (Figure 4). A high SD value in RNA yield could be the result of the heterogeneity occurred when samples were processed such as in the step of plasma aliquote, and so on.

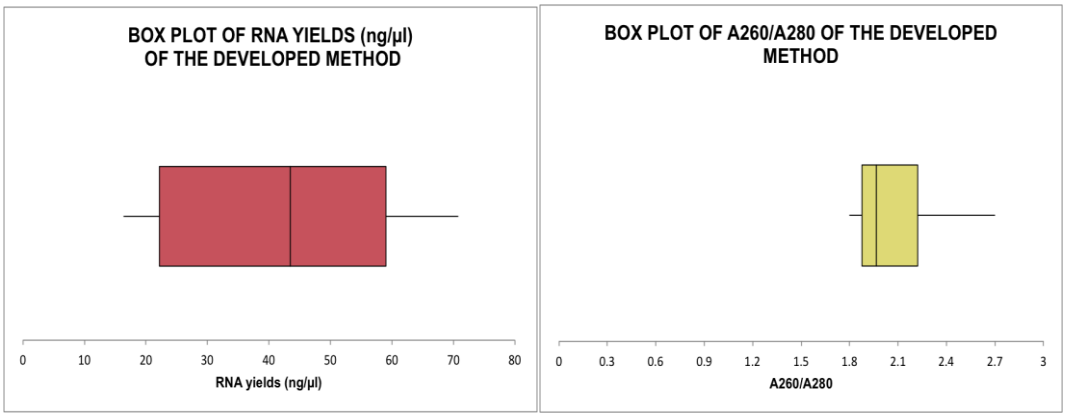

Figure 4. Box plot of quantity and purity ratio of RNA extracted by the newly developed method

CONCLUSION

As the therapeutic use of miRNAs has been proven these days, there is an increasing demand for the development of an efficient small RNAs isolation method from biological specimens, especially plasma samples. Nonetheless, the technical aspects of small RNAs extraction are still in its infancy stage. This study has introduced a new efficient technique that can isolate small RNA in a high quantity and quality, which is beneficial for medical research. With the use of $MgCl_2$ instead of LiCl, this alternative protocol could maximize the amount of small RNA extracted while ensuring its high purity. Furthermore, the newly developed method not only overcomes the disadvantages of isolation kit such as high cost and long delivery time but also is comparable in terms of extraction efficiency.

However, due to the time limitation, the expression of small RNAs isolated using this method has not been checked. We suggest that more work should be carried out to confirm the potential of the developed protocol in

extracting small RNAs. The quantitative real-time PCR, thereafter, will be used to analyze the RNA expression. Also, the method should be performed in a larger sample size to confirm its stability.

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XÂY DỰNG PHƯƠNG PHÁP TÁCH CHIẾT RNAS NHỎ TỪ MẪU HUYẾT TƯƠNG

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TÓM TẮT

Trong những năm gần đây, phương pháp định lượng miRNA đã và đang nhận được nhiều quan tâm trong chẩn đoán trước và điều trị ung thư. Mặc dù các bộ kit thu mẫu RNA được sử dụng rất phổ biến, chúng tốn nhiều chi phí và gây nhiều hạn chế cho các phòng thí nghiệm quy mô nhỏ. Từ đó, các kỹ thuật khác bao gồm sử dụng Trizol được đề xuất như một giải pháp hữu hiệu để thay thế. Tuy nhiên, các nghiên cứu cho thấy rằng LiCl trong phương pháp này không thể kết tủa các RNA nhỏ một cách hiệu quả như $MgCl_2$. Trên cơ sở đó, nghiên cứu này đặt mục tiêu phát triển và tối ưu hóa phương pháp một cách hiệu quả hơn trong việc tách chiết các RNA nhỏ. Sau khi chọn nồng độ $MgCl_2$ phù hợp nhất, nghiên cứu thực hiện so sánh giữa việc sử dụng $MgCl_2$, LiCl và bộ kit thu mẫu. Hiệu năng của các phương pháp được xác thực thông qua kiểm tra chất lượng của các RNA tách chiết bằng cách sử dụng phép đo nồng độ và tỷ lệ hấp thụ A260/A280. Kết quả cho thấy rằng sự kết tủa của các RNA nhỏ sử dụng hỗn hợp 0,5M $MgCl_2$ và ethanol mang lại năng suất cao nhất, cao hơn năm lần và mười lần so với LiCl và bộ kit tách chiết. Ngoài ra, so sánh giữa phương pháp mới được phát triển với bộ kit tách chiết cho thấy rằng phương pháp này không chỉ chiếm ưu thế về nồng độ RNA thu được mà còn về mức độ tinh sạch của mẫu. Như vậy, phương pháp trên hứa hẹn sẽ là một cách thay thế hiệu quả để tách chiết các RNA nhỏ, từ đó giảm sự phụ thuộc vào việc sử dụng bộ kit thu mẫu.

Từ khóa: miRNA, plasma samples, $MgCl_2$, LiCl, isolation kit.

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