Accelerated and efficient method for isolating microRNA from human blood plasma

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Abstract: The study is dedicated to solving the problem of microRNA extraction from human blood for further use of the microRNA profile in the diagnosis of various diseases. The aim of the work is to choose an effective, accelerated method for the isolation of microRNA from human blood plasma, sufficient in quantity for diagnosing in clinical practice. Comparison of three methods for obtaining microRNA was carried out: 1) using a silicon oxide-based QIAamp RNA Blood Mini Kit column, 2) phenol-chloroform extraction using TRIzol LS Reagent and 3) phenol-chloroform extraction using TRIzol Reagent. It was established that for the most complete isolation of total RNA from human blood plasma in order to obtain microRNA, TRIzol LS Reagent can be efficiently used. Because its use allows obtaining microRNA in less time – 30–40 min, and in an amount sufficient for clinical analysis (total RNA – up to 0.116 ng/μl, and microRNA – 350.3 picograms/μl), and also with the least amount of concomitant messenger RNA and long non-coding RNA. Using TRIzol LS Reagent allows you to increase the output of miRNA by 2.7 times compared with the use of TRIzol Reagent.

Keywords: microRNA, blood plasma, diagnostics, biomarker

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Ускоренный и эффективный метод выделения микроРНК из плазмы крови человека

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Резюме: Исследование посвящено решению проблемы экстракции микроРНК из крови людей для дальнейшего использования профиля микроРНК в диагностике различных заболеваний. Целью работы являлся выбор эффективного, ускоренного метода выделения микроРНК из плазмы крови человека, достаточного по количеству для проведения диагностики в клинической практике. Проведено сравнение трех методов получения микроРНК: 1) с применением колонки на основе оксида кремния QIAamp RNA Blood Mini Kit, 2) фенол-хлороформной экстракции с помощью TRIzol LS Reagent и 3) фенол-хлороформной экстракции с применением TRIzol Reagent. Установлено, что для наиболее полного выделения из плазмы крови человека тотальной РНК с целью получения микроРНК эффективно использовать реактент TRIzol LS Reagent, поскольку его применение позволяет получить микроРНК за меньшее время – 30–40 мин, и в количестве, достаточном для проведения клинического анализа (точальной РНК – до 0,116 нг/мкл, и микроРНК – 350,3 пикограмм/мкл), а также с наименьшим количеством сопутствующих матричных РНК и длинных некодирующих РНК. Использование TRIzol LS Reagent позволяет увеличить выход микроРНК в 2,7 раза по сравнению с применением реактента TRIzol Reagent.

Ключевые слова: микроРНК, плазма крови, диагностика, биомаркер
INTRODUCTION

Currently, one of the most promising objects for the study of the human genome is short non-coding RNA (ncRNA), since these molecules perform important functions in the cell. For example: 7SL RNAs regulate protein translation and post-translational transport, as well as playing a major role in the regulation of transcription. Small nuclear RNA involved in the maturation of rRNA. Small interfering RNAs (miRNAs) inhibit the expression of mobile elements and participate in the regulation of gene expression, which also includes microRNAs involved in the regulation of gene expression [1]. By their chemical nature, microRNA comprises a class of short, non-coding 5'-phosphorylated RNA, 19–24 nucleotides in length. MicroRNAs have been detected in all major cellular organelles, including the nucleus, nucleolus and mitochondria, where they perform the function of post-transcriptional regulation of genes. MicroRNAs affect a messenger RNA (mRNA) target. Post-transcriptional regulation of mRNA is of great importance for maintaining the balance of proteins in cells, which is necessary for the normal functioning of the body [2].

Since the microRNA content of human blood plasma acts as biomarkers for various diseases, it is very important to obtain this information for diagnostic purposes. Consequently, obtaining this information from human blood plasma in an accelerated mode and in a quantity sufficient for diagnosis is of great interest and can make a significant contribution to the early detection of such severe conditions as diabetes, cardiovascular and oncological diseases [3–5]. MicroRNA molecules can also perform a prognostic function, since they regulate cellular processes in higher animals in normal and pathological conditions [3–5]. The discovery of extracellular microRNAs in serum, plasma and other body fluids has provided a new potential source of non-invasive biomarkers for cancer and other diseases [6]. MicroRNAs have a number of useful characteristics that are inherent to biomarkers: not only are they specific for different types of tissues, but they are also capable of revealing the presence of a disease in its early stages and sensitive to changes that occur during illness. The high stability of the molecule is particularly noteworthy; moreover, MicroRNA analysis is non-invasive [7–9].

Despite the wide range of studies devoted to determining changes in microRNA profiles during the development of certain diseases, technical problems involved in their isolation continue to hinder the use of microRNA in clinical diagnostics. One of the biggest problems for the study of extracellular microRNAs is their relatively low concentration in serum and plasma samples. To date, there are several microRNA extraction kits for obtaining small non-coding RNA from blood, each of which has its own advantages and disadvantages. However, the most commonly-used isolation approach extracts microRNA from total RNA [10–18].

The authors of the article [14] evaluated various methods for the extraction of microRNA from human serum. The following reagents were used: miRNasy Mini Kit (Qiagen, USA), TRIzol Reagent (Invitrogen, Life Technologies, USA), TRIzol LS Reagent (Invitrogen), and the mirVana miRNA reagent kit (Ambion, Life Technologies, Thermo Fisher Scientific, USA). It has been shown that microRNA isolation using TRIzol Reagent is most suitable for large-scale experiments, allowing the maximum recovery of low molecular weight microRNA to be achieved. When comparing TRIzol Reagent with column isolation methods, it was found that the Qiagen miRNasy Serum kit/Plasma Kit allows more concentrated microRNA to be obtained for study purposes.

In the study [15], the authors used a standard extraction protocol using the TRIzol LS Reagent (ThermoFisher Scientific, USA) to isolate plasma microRNAs. For this purpose, they also used the miRNasy mini kit (Qiagen, Germany) or mirVana PARIS (ThermoFisher Scientific, USA), as well as the miRNA kit (Norgen Biotek, Canada) for the isolation of microRNA from cells, tissues and biological fluids. The results obtained on the use of three different sets of reagents with columns compared with the use of TRIzol LS Reagent for isolating microRNAs showed that methods using columns are more efficient and reliable, as well as demonstrating a higher reproducibility of the results of extracellular microRNA isolation. When using TRIzol LS Reagent based on phenol-chloroform extraction, the authors were not able to achieve the same efficiency of microRNA extraction despite the modifications introduced by this method, but that the mirVana PARIS kit is capable of obtaining a higher microRNA content.

The authors of the study [18] compared different methods for extracting RNA from various biological fluids and cells for subsequent isolation of microRNA. In particular, the authors used phenol–TRIzol LS Reagent, two sets of reagents with columns (miRCURY RNA Isolation and miRCURY), as well as combined phenolic and column sets (miRNasy Mini Kit). Evaluation of microRNA recovery was performed using capillary electrophoresis and real-time quantitative reverse tran-
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It is shown that miRCURY and miRNeasy are equally effective for the extraction of RNA and are superior to TRIzol LS Reagent. However, the use of Trizol LS Reagent made it possible to obtain the largest amount of microRNA in the small RNA fraction. At the same time, the low purity of the RNA obtained using Trizol LS Reagent did not give satisfactory qRT-PCR results. The authors of another study reported that Trizol LS Reagent was more effective than mirVana PARIS (combined phenol and column method) in the extraction of microRNA from blood plasma [6].

As can be seen, at the present time there is no consensus on the best approach to solving the issue of isolating microRNA from blood plasma. Although there are many works on plasma microRNA studies, their results are not easily comparable, and, as yet, there is no standardised and effective method for isolating microRNA from blood plasma [6].

The aim of this study was to select an effective, accelerated method for the isolation of microRNA from human blood plasma, sufficient in quantity for diagnosis within clinical practice.

EXPERIMENTAL PART

Venous blood sampling and sample preparation. Samples of venous blood from healthy young people who agreed to participate in the research were used for the study. Blood was collected in BD Vacutainer tubes with EDTA (Becton Dickinson, USA) and centrifuged using an Eppendorf 5702R centrifuge (Eppendorf, Germany) applying 2000 g for 5 minutes at 4 °C. Following centrifugation, the upper plasma layer was transferred into clean 1.5 ml tubes free from RNases.

Isolation of total RNA. In order to isolate total RNA, the following methods were applied:

1) RNA purification using a silicon oxide membrane without organic extraction with phenol-chloroform, by means of the QIAamp RNA Blood Mini Kit reagent (QIAGEN, Germany);
2) phenol-chloroform extraction using TRIzol Reagent (TR) (ThermoFisher Scientific, USA);
3) phenol-chloroform extraction using TRIzol LS Reagent (TLSR) (ThermoFisher Scientific, USA).

With the first RNA purification method, 1 part of human whole blood was mixed with 5 parts of buffer. Next, it was incubated for 15 min on ice and centrifuged at 4000 g for 10 min at 4 °C and the supernatant was completely removed. Buffer EL from the reagent kit was added to the cell sediment. The mixture was then centrifuged at 4000 g for 10 min at 4 °C and the supernatant was completely removed. The lysate was added to the column and centrifuged for 2 minutes at maximum speed. One volume of 70% ethanol was added to the lysate which was passed through the column and transferred to a new column. Then it was centrifuged for 15 s at 8000 g. To wash RNA, 700 μl of RW1 buffer was added to the column, then centrifuged for 15 s at 8000 g. Then 500 μl of RPE buffer was added to the column and centrifuged for 15 s at 8000 g. Also 500 μl Buffer RPE was added and centrifuged at 20000 g for 3 minutes. To obtain RNA, 30–50 μl of water was added to the column and centrifuged for 1 min at 8000 g for elution.

The second method — using TRIzol Reagent, after separation of blood plasma macromolecules using chloroform (the ratio of the volume of TRIzol Reagent with the sample to chloroform is 5:1), the fraction containing the total blood plasma RNA was selected. Purification of total plasma RNA was performed with the addition of 0.5 ml of 100% isopropanol (the ratio of RNA and isopropanol was 1:1) and subsequent centrifugation for 10 min at 15000 g and 4 °C. The supernatant was removed into a precipitate containing RNA and 75% freshly prepared ethanol ("Tat-spiritrom", Russia) was added. Then RNA was precipitated for 10 minutes at 20000 g and 4 °C. The supernatant was removed, then the RNA was dried and dissolved in water that was free of nuclease.

Using the third method — with TRIzol LS Reagent, operations for isolating total RNA were performed similarly to the second method.

Analysis of the amount and composition of RNA in samples. Quantitative analysis of total blood plasma RNA was performed using the fluorometric method on a Qubit 2.0 instrument (ThermoFisher Scientific, United States).

Agilent RNA 6000 Pico Kit reagents (Agilent Technologies, USA) were used to analyse the total quantity of RNAs. The composition of microRNAs was studied using the Agilent Small RNA Kit (Agilent Technologies, USA) reagent kit with chips. Samples with RNA were measured on an Agilent 2100 Bioanalyzer (Agilent Technologies, United States) according to the manufacturer’s instructions.

1. Statistical analysis. After analysing the size and quantity of the RNA contained in the samples, the data obtained from the bioanalyzer was subjected to statistical evaluation. Statistical processing of the results obtained during the three repetition of the experiment for each of the presented methods was standardly performed using Microsoft Excel 2016 and the built-in “Standard deviations” function package.

RESULTS AND DISCUSSION

When comparing the production of microRNA by various methods, the following is established.

The experiment conducted using the first method using a whole blood separation column allowed whole blood to be used without prior separation into fractions. However, following purification, RNA was

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obtained in an amount insufficient for further analysis; the time taken to complete the work was around 1 hour.

Second and third methods. Using TRIzol Reagent, neither 18S nor 28S ribosomal RNA was detected in the samples. In the case when TRIzol Reagent was used, it was shown that RNA is present in the samples in the range from 25 to 4000 nucleotides (Fig. 1), which indicates the presence of both microRNA and mRNA, as well as long non-coding RNA in the sample in significant quantities. Furthermore, the concentration of total RNA when isolated using TRizol Reagent is lower than when using TRizol LS Reagent (Fig. 2).

The advantage of using TRizol LS Reagent is that the samples do not contain 18S or 28S ribosomal RNA due to their higher molecular weight. It was established that the bulk of the obtained RNA contains from 25 to 500 nucleotides, including microRNA. The samples exhibit a low content of messenger RNA or long non-coding RNA with a size of 500 to 4000 nucleotides (Fig. 3).

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**Fig. 1. Size analysis of human blood plasma RNA obtained purified using the "TRIzol Reagent" isolation procedure**

**Рис. 1. Анализ размера полученных РНК плазмы крови человека, очищенных с использованием методики выделения «TRIzol Reagent»**

**Fig. 2. The quantitative content of total RNA in the human blood plasma samples**

**Рис. 2. Количество содержание тотальных РНК в исследуемых образцах плазмы крови человека**
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Using TRIzol LS Reagent, a high yield of total RNA is confirmed, and consequently, microRNA. When determining the concentration, it was found that, by using TRIzol Reagent, the concentration of total RNA was 0.043 ng/μl, whereas using TRIzol LS Reagent, the concentration was 0.116 ng/μl. Thus, TRIzol LS Reagent isolates total RNA from blood plasma 2.7 times more effectively; therefore, producing more microRNA – about 50% of small RNAs (see Fig. 2). When total RNA was isolated using TRIzol Reagent, the concentration of microRNA was 214.3 picogram/μl; when using TRIzol LS Reagent – 350.3 picogram/μl.

Thus, the analysis of total RNA and microRNA extraction from human blood plasma using TRIzol LS Reagent showed that this method made it possible to obtain a greater concentration of small RNA and microRNA plasma and a greater amount of isolated total RNA compared to using TRIzol Reagent or QIAmp mini kit.

CONCLUSIONS

1. Using TRIzol LS Reagent makes it possible to increase the output of microRNA by 2.7 times compared with the use of TRIzol Reagent.

2. For the most complete isolation of total RNA (up to 0.116 ng/μl) and microRNA (350.3 picograms/μl) from human blood plasma, it is efficient to use TRIzol LS Reagent, since it allows microRNA to be obtained in a shorter time (30–40 min) and in a sufficient quantity for clinical analysis. Furthermore, the obtained microRNAs contain the smallest amount of concomitant messenger RNA and long non-coding RNA compared with use of the QIAmp mini kit.

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**Contribution**

Irina A. Letova, Saidamalxon A. Madumarov, Maria A. Sysoyeva, Rayhan Z. Shah Mahmud carried out the experimental work, on the basis of the results summarized the material and wrote the manuscript. Irina A. Letova, Saidamalxon A. Madumarov, Maria A. Sysoyeva, Rayhan Z. Shah Mahmud have equal author’s rights and bear equal responsibility for plagiarism.

**Conflict of interests**

The authors declare no conflict of interests regarding the publication of this article.

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