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OPTIMIZATION OF PCR PURIFICATION USING SILICA-COATED MAGNETIC BEADS

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ABSTRACT

Purification of nucleic acids is still an important step in molecular genetic research. The development of whole genome sequencing technologies has increased the requirements for the purity of the nucleic acids used, and also required the selection of DNA fragments by size. Buffer systems that contain PEG/NaCl solutions and silica-coated magnetic beads allow to purify nucleic acids and selectively sorb certain sizes of DNA. In this article, we present a simple protocol for the purification of PCR products with the ability to absorb the required DNA molecules. It was determined that the use of an optimized PEG / NaCl buffer system with magnetic silica gel in a ratio of 1.5: 1 with a PCR product allows to get rid of DNA fragments 100 and less base pairs (bp), as well as other contaminants, while maintaining this is more than 90% of the DNA in solution. The ratio of 0.35: 1 allows for high-affinity sorption of DNA molecules larger than 400 bp. The practical use of the obtained data allows us to improve the quality of sequencing without increasing the cost of research.

Key words: silica-coated magnetic beads, purification, DNA, PCR products.

INTRODUCTION

Nucleic acid purification methods that are used in genetic analysis and identification of biological objects play a crucial role in research in the field of life sciences. [1]. For diagnostic and research purposes, it is necessary to isolate nucleic acids from various objects including bacteria, virus-containing liquid, tissues and exudates of higher organisms. To isolate nucleic acids, there is a huge selection of commercially available kits and open protocols that make it possible to obtain highly purified nucleic acids from impurities of cell walls, proteins and chemical compounds [2,3].

Another important task of molecular biology is to purify PCR products for subsequent cloning or sequencing including preparing libraries for sequencing on high performance sequencers. [4]. Two main factors affect the quality of the resulting sequences: the concentration, integrity, and purity of the DNA matrix; effective removal of primer residues, unbound deoxynucleotide triphosphates, proteins and chemical compounds [5]. In addition, to clear libraries for whole genome sequencing, it is necessary to select PCR products by size, removing non-specific low-molecular and high-molecular DNA fragments formed during the demirization of primers and DNA fragmentation. Methods for cleaning PCR products can often be expensive, time-consuming, and can lead to partial or complete loss of the original DNA template. The determination of a nucleic acid purification technique may depend on several factors,

such as cost, operating time, the need for high-performance processing, the quality of sequence data, and user preferences. Numerous approaches are available for purification of PCR products, including alcohol precipitation [6], affinity chromatography [7], size exclusion chromatography methods [8-10], proprietary kits, and the use of paramagnetic particles or beads [11-12]. Among these methods, the method of cleaning nucleic acids with paramagnetic particles coated with silica gel has a number of advantages. The use of magnetic silica gel reduces operating time and costs and increases the integrity of the DNA. [13]. Also, magnetic extraction does not use harmful organic solvents, the protocols are simple and allow you to automate the processes. [14]. Magnetic silica gel has been widely used in cleaning up "libraries" for whole genome-sequencing, as it has several advantages: unlike cleaning libraries on spin column, it does not require a centrifugation stage, which allows you to automate and scale the cleaning process [15]; get rid of low molecular weight DNA fragments [16]; get rid of long DNA molecules [17].

Magnetic silica gel has a ferrite core coated with silica or carboxylic polymer, which eliminates the oxidation of iron and prevents the loss of its properties, and also facilitates the sorption of nucleic acids. These properties of magnetic silica gel allow reversible binding of nucleic acid under conditions of dehydration and in the presence of a strong magnet can be safely immobilized during several stages of washing and manipulation [18]. The particle structure and composition of the solution can be modified to selectively adsorb nucleic acids depending on their type and size. These properties were used to develop purification methods based on a three-stage process of binding, washing and elution. In an optimized binding solution, the particles selectively adsorb nucleic acids from complex mixtures. An external magnetic field is used to remove particles and adherent target nucleic acid from unbound material. Flushing particles eliminates residual contamination. The purified target nucleic acid is then eluted, ready for subsequent applications. This technology was developed as an alternative to vacuum filtration and centrifuge-based cleaning formats to ensure high sample processing performance. Commercially available kits are expensive and significantly increase the cost of the sequencing process. In this regard, many groups develop their protocols using ready-made or self-prepared silica gels [19]. However, it is necessary to optimize the conditions for cleaning nucleic acids for a certain type of magnetic silica gel.

In this article we present an optimized method of nucleic acid purification that demonstrates quality results comparable to data obtained using more expensive commercial kit.

Materials and methods

In this work was used a sample of *P. multocida* DNA isolated from saiga fallen during the epizootic in 2016, from the collection of "National Reference Center for Veterinary Medicine". Cultivation of the *P. multocida* strains was carried out on meat peptone agar (HiMedia, India). DNA was isolated using the QIAamp DNA Mini Kit manufactured by QIAGEN (USA) according to the manufacturer's instructions. DNA concentration was determined using a NanoDrop1000 spectrophotometer. We also used RNA samples isolated using the GeneJET Viral DNA and RNA Purification Kit (ThermoScientific) from patients diagnosed with SARS-Cov2 coronavirus infection.

PCR amplification. Amplification of the 16S rRNA gene fragment was performed with universal primers 8f 5 'and 806R [20] in a total volume of 30 ul. The PCR mixture contained 2 ng of *P. multocida* DNA, 1U Taq DNA Polymerase (ThermoScientific), 0.2 mM of each dNTP, 1xPCR KCl buffer, 2.5 mM MgCl2, 30

pmol of each primer. Excess primer was used as a purification control. The PCR program: denaturation at 95°C for 5 min, 30 cycles (95°C for 35 s, 55°C for 30 s and 72°C for 60 s) and final elongation at 72°C for 5 min. PCR was performed using a MasterCycler Gradient thermal cycler (Eppendorf).

Amplification of SARS-Cov 2 virus fragments was performed in a total volume of 25 µl using a SuperScript III One-Step RT-PCR Platinum Taq Hi-Fi Set (Invitrogen), according to the manufacturer's instructions. Each reaction contained 10 pmol of direct and reverse primers and 5 µl of RNA. The PCR program: reverse transcription stage at 56°C for 30 min; denaturation of 94°C for 3 min; 35 cycles (94°C for 15 s, 58°C for 30 s, 68°C for 2 min) and final elongation at 68°C for 5 min. PCR was performed using a MasterCycler Gradient thermal cycler (Eppendorf).

Enzymatic purification. Enzymatic purification of PCR products for sequencing was performed using Exonuclease I (ThermoScientific) and alkaline phosphatase (Shrimp Alkaline Phosphatase, ThermoScientific) [21].

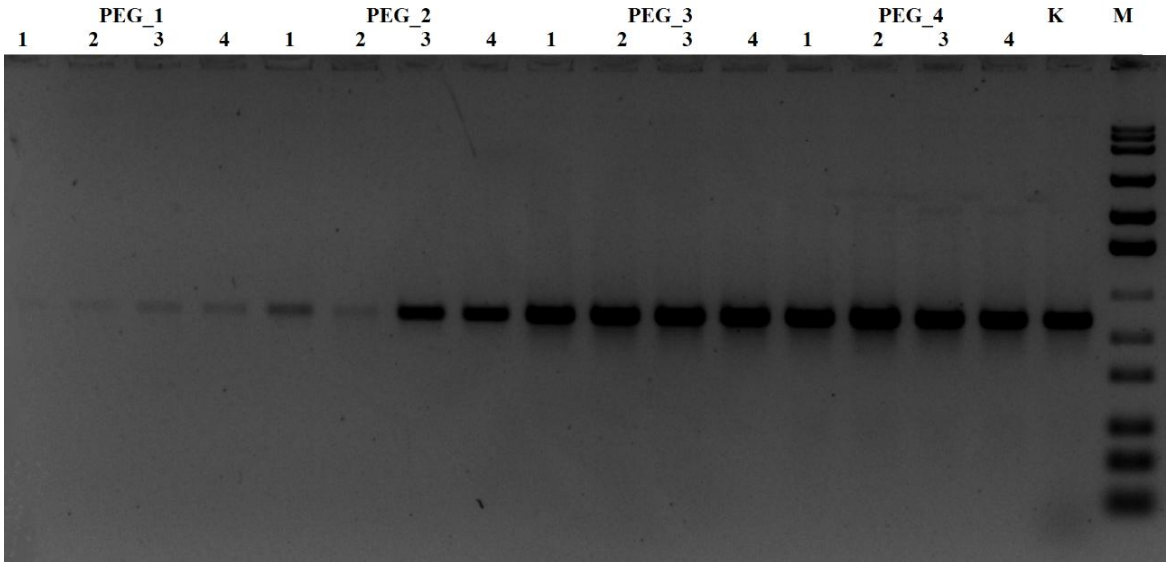
Purification using magnetic particles coated with silica gel. Optimization of PCR purification of products using magnetic particles coated with silica gel. For this purpose, were prepared 4 variants of binding buffer systems containing different concentrations of PEG 8000 (polyethylene glycol) (AppliChem) and NaCl: PEG_1 (9.1% PEG 6000 in 2 M NaCl), PEG_2 (15% PEG 8000 in 1.5 M NaCl), PEG_3 (15% PEG 8000 in 2 M NaCl), PEG_4 (20% PEG 8000 in 2.5 M NaCl). Silica gel particles have abrasive properties and, when rubbed against each other, form nanosized impurities. Therefore, before using magnetic silica gel, it must be washed with sorption buffer (10mM Tris-HCl, ph 8.0 1 mM EDTA, pH 8.0) to remove fine impurities and optimize pH. Magnetic silica gel (5% molbiol.ru) was added immediately before use to a concentration of 0.1 %. As eluting solutions, 1x TE pH-9.2 (10 mM Tris-1mM EDTA), deionized water, 10 mM Tris HCl pH-8, 1x TE buffer pH 8.0 were used.

The products of 20 PCR 16S rRNA reactions of *P. multocida* were mixed and transferred 20 µl into 25 tubes, 24 of which were used to verify purification using various concentrations of PEG and elution solutions, and 1 sample was a control. To 20 µl of the PCR product was added 30 µl of PEG solution with magnetic silica gel, stirred on a shaker for 2 minutes at 1800 rpm. Incubated at room temperature for 10 minutes. After the stage of DNA binding with magnetic particles, the plate was placed on a magnetic tripod (00347252 Magnetic Stand-96, Invitrogen,) for 2 minutes, after the solution was clarified, the supernatant was removed. The magnets were washed twice with 100 µl of 70% ethanol. The product was eluted in 20 µl for 10 minutes at 60°C, with shaking after 5 minutes of incubation on a shaker for 1 minute at 1600 rpm. Then the plate was placed on a magnetic tripod, the purified PCR product was taken and electrophoretic analysis was performed in 1.5% agarose. Concentration was measured on a Qubit® 2.0 fluorimeter (Invitrogen) and with the dsDNA HS Assay Kit (Invitrogen).

Determination the dependence of ratio of PEG solutions on the size of adsorbing fragments. For this purpose, marker DNA was purified with fragments from 100 to 1000 bp (100 bp step) with a variable ratio of the purified product to the PEG/NaCl solution. The ratio of the volume of the PEG solution to the volume of the DNA marker ranged from 0.35 to 1.8. A commercially available magnetic particle cleaning solution called AMPure XP (Beckman Coulter) was used as a control in an identical range.

RESULTS

Determination of the optimal PEG/NaCl solution. To evaluate the efficiency of purification, a single protocol was used with the ratio of the PCR product and PEG solutions 8000 / NaCl with silica-coated magnetic beads 1: 1.5. The highest adsorption efficiency of PCR products was observed in test tubes with 20% PEG 8000 and 2.5 M NaCl (figure 1). In the phoregram with this solution, the highest luminescence intensity of the target PCR product was observed, but there was no low molecular weight nucleic acid (up to 100 bp). A quantitative assessment (table 1) confirms the highest efficiency of purification of PCR products with a solution of magnetic silica gel and 20% PEG 8000 and 2.5 M NaCl. The total DNA output in protocols with 9.1% PEG 6000 in 2 M NaCl, 10% PEG 8000 in 1.5 M NaCl and 15% PEG 8000 in 2.0 M NaCl compared with 20% PEG 8000 in 2.5 M NaCl was on average lower by 97.2%, 76.7% and 29.2%, respectively. Among the eluting solutions, 1x TE pH-8.0, and 10 mM Tris HCl, pH-8 was most effective.



PEG_1 - 9.1% PEG 6000/2 M NaCl; PEG_2 - 10% PEG 8000/1.5 M NaCl; PEG_3 - 15% PEG 8000/2.0 M NaCl; PEG_4 - 20% PEG 8000/2.5 M NaCl.
Eluting solutions: 1 - 1xTE –pH 9.2, 2 -Dionized water, 3-10 mM TrisHCl pH 8.0, 4-1xTE –pH 8.0
M- # SM1293, molecular weight marker from 100 to 10,000 bp
Fig.1. Purification quality of PCR products with different concentrations of PEG/NaCl

Table 1. Quantification of effectiveness the PCR products purification

Purification method	Concentration during elution with various solutions ng/µl			
	10 mM TrisHCl pH 8.0	1xTE –pH 8.0	1xTE –pH 9.2	Dionized water
9.1% PEG 6000/2 M NaCl	0.688	0.717	0.448	0.353
10% PEG 8000/1.5 M NaCl	5.53	7.03	4.53	1.71
15% PEG 8000/2.0 M NaCl	16.8	17.7	12.5	10.1
20% PEG 8000/2.5 M NaCl	20.3	20.5	20.0	19.8
Note: Conc (K) – 21.6 ng/µL				

Estimation the ratio of PEG 8000 / NaCl with sizes of adsorbed fragments. Based on the results of optimizations, in further work we used PEG 8000 20% and 2.5 M NaCl with 0.1% silica-coated magnetic beads. As an eluting solution, we used 1xTE, pH-8. The optimized composition of PEG 8000 / NaCl with magnetic silica gel in a ratio of 0.35 allows sorption of fragments from 500 bp, a ratio of 0.65 to 1.2 - from 200 bp (figure 2). The ratio of 1.8:1 adsorb fragments from 100 bp. Using a commercial AM Pure XP solution, a ratio of 0.35: 1 led to almost complete loss of a marker of all lengths, a ratio of 0.65: 1 and 0.8: 1 adsorb molecules from 300 bp, while a ratio of 1.2 : 1 and 1.8: 1 begins to adsorb DNA fragments with 200 bp.

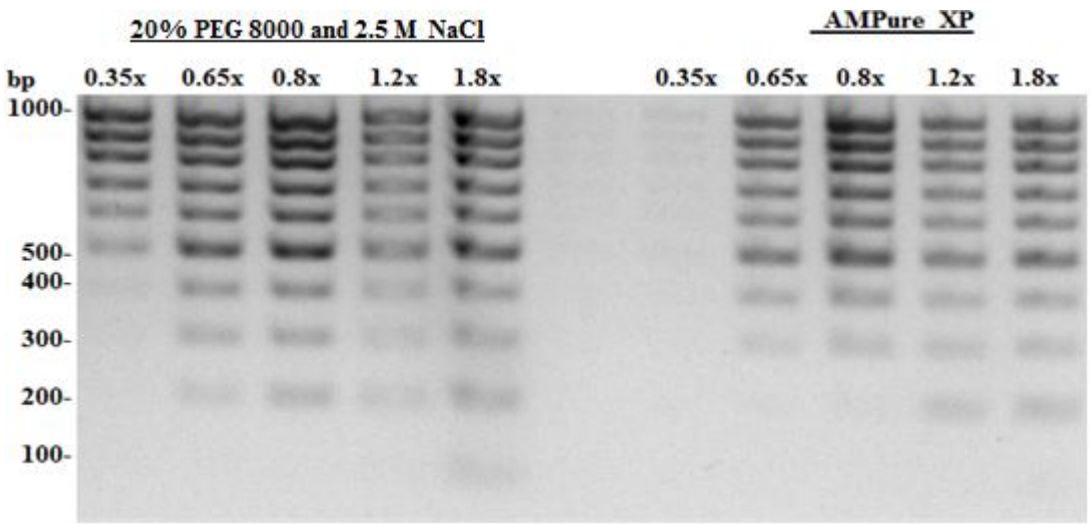
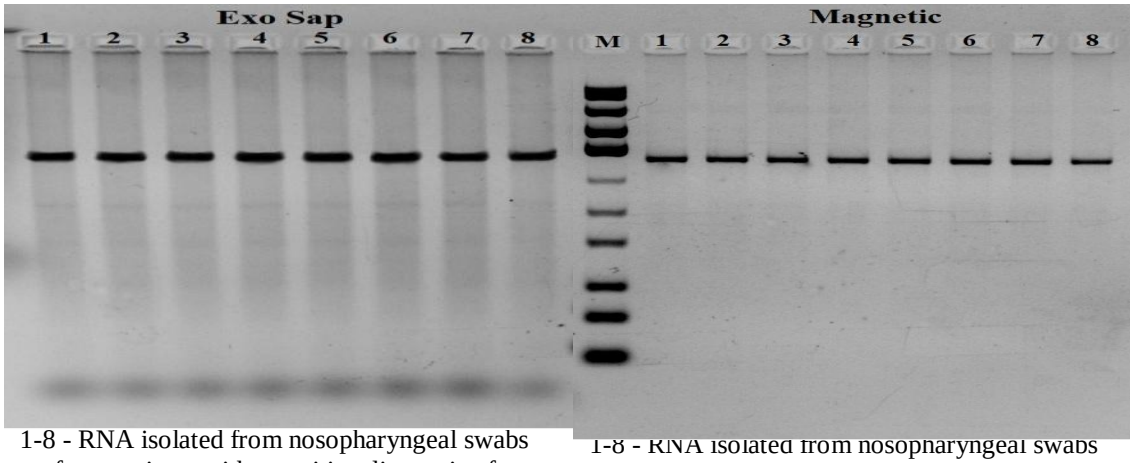


Fig. 2. Assessment the ratio of PEG 8000/NaCl with sizes of adsorbed fragments

Purification of PCR products for "Sanger" sequencing method. PCR products amplifying SARS-Cov2 fragments were used as the initial template. Amplification was performed on RNA samples isolated from nosopharyngeal swabs from patients with a positive diagnosis of coronavirus infection. High concentrations of non-targeted RNA generated non-specific low molecular weight fragments and smears (figure 3A), which led to a deterioration in the quality of sequencing PCR products purified by the enzymatic method (figure 4A). Using an optimized method with a ratio of 0.35:1 made it possible to significantly clear PCR products from low-molecular compounds (figure 3B), thereby improving the quality of sequencing (figure 4B).



1-8 - RNA isolated from nosopharyngeal swabs from patients with a positive diagnosis of coronavirus infection
M- # SM1293, molecular weight marker from 100 to 10,000 bp

Fig.3A. PCR products after enzymatic purification

1-8 - RNA isolated from nosopharyngeal swabs from patients with a positive diagnosis of coronavirus infection
M- # SM1293, molecular weight marker from 100 to 10,000 bp

Fig. 3B. PCR products purified by magnetic silica beads

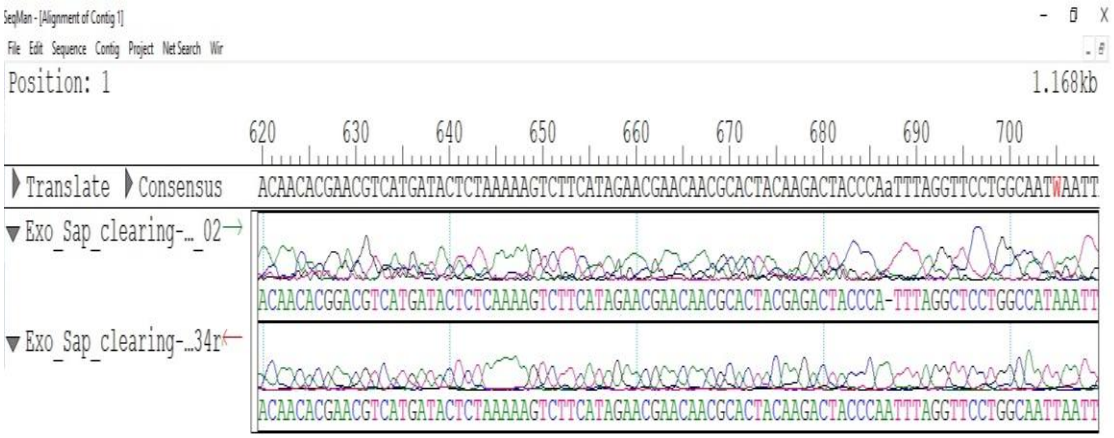


Fig. 4A. The result of sequencing of PCR products after enzymatic purification

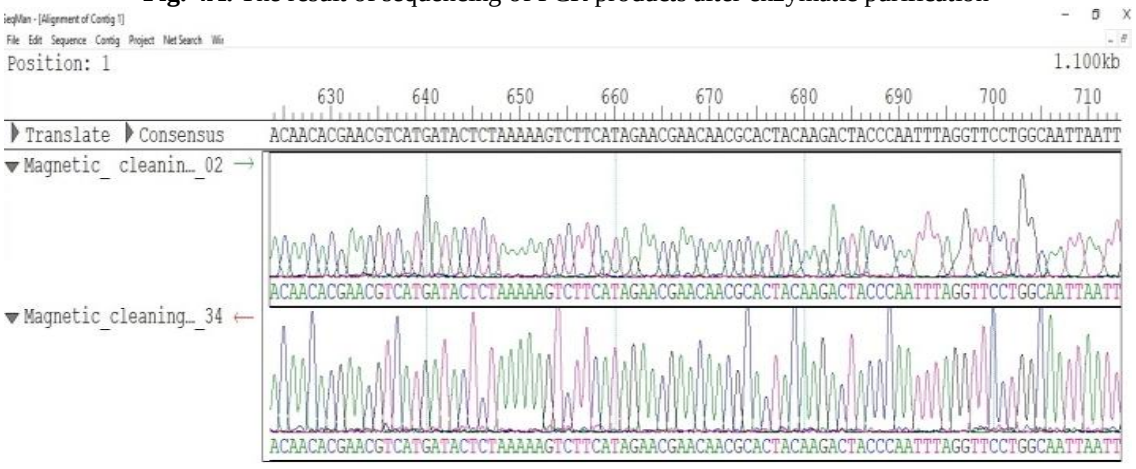


Fig. 4B. Result of sequencing of PCR products with magnetic silica beads

DISCUSSION

The use of magnetic particles for the isolation and purification of nucleic acids

began in the 90s of the last century [22]. Currently, many sets of nucleic acid isolation and purification of PCR products have appeared on the market. Nevertheless, despite the simplicity of the methodologists, their cost is still high. In this article, we describe a protocol for the purification of PCR fragments using reagents standardly available in research laboratories. The developed protocol in its technical characteristics is not inferior to commercial analogues.

Magnetic silica gel is a particle of iron oxide coated with silica or polymer, which is why it has a magnetic moment and can move when exposed to a magnetic field. Creating a magnetic field at the edge of the test tube leads to the formation of a clot of magnetic silica gel at the place where the magnetic field is applied. This ability provides a number of advantages for methods based on magnetic particles in comparison with standard methods based on spin columns, silica gel, and nucleic acid precipitation. The absence of a centrifugation step allows to automate the process and reduce the risk of cross-contamination [23]. It allows one to isolate a product from large volumes with a low DNA concentration without increasing the process execution time [24, 25].

Size fractionation of DNA molecules is an important advantage that has found application in the preparation of samples for whole genome sequencing.

DNA size selection method is a two-step adsorption of DNA on magnetic particles. For this, at the first stage, high affinity binding of large DNA fragments with a length of more than 800 bp occurs, which is achieved by adding a PEG / NaCl solution with paramagnetic particles in a ratio of 0.5: 1 to the sample volume. For the second stage, the supernatant is transferred to a new tube into which PEG / NaCl with paramagnetic particles is added in a ratio of 0.7: 1. As a result, sorption of DNA fragments of more than 300 bp occurs. After removal of unbound short DNA fragments and washing of magnetic particles, the sorbed fragments are eluted, which allows obtaining ready-made libraries of 300-800 bp in size. [26]. We used various concentrations of PEG8000 and NaCl with a constant concentration of magnetic silica beads. The use of the previously described concentrations of PEG8000 and NaCl [27, 28] led to large losses of target fragments. Only an increase in the concentration of PEG8000 to 20% and NACl to 2.5 mM led to a decrease in losses during the purification of PCR products. The optimized buffer, when used in a ratio of 1.5: 1 to the volume of DNA solutions, made it possible to purify PCR products from primers of dimers, salts, and other contaminating components of the reaction, retaining from 91.6 to 94.9% of DNA in solution.

Applying different ratios of volume of buffer systems with magnetic silica to the volume of the sample allowed to selectively adsorb DNA fragments by size. Thus, the ratio of 0.35: 1 made it possible to selectively adsorb fragments of more than 400 bp, which was used in the purification of PCR fragments containing low molecular weight smears. Purification using magnetic particles significantly improved Sanger sequencing results.

CONCLUSIONS

An optimized protocol allows purification of PCR products and is not inferior to commercial analogs in results.

Acknowledgements

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ОПТИМИЗАЦИЯ МЕТОДА ОЧИСТКИ ПЦР МАГНИТНЫМ СИЛИКАГЕЛЕМ

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АБСТРАКТ

Очистка нуклеиновых кислот по-прежнему является важным шагом в молекулярно-генетических исследованиях. Развитие технологий секвенирования всего генома ужесточила требования к чистоте используемых нуклеиновых кислот, а также потребовало проводить селекцию фрагментов ДНК по размерам. Буферные системы, содержащие растворы ПЭГ / NaCl и магнитные шарики, покрытые силикагелем, позволяют очищать нуклеиновые кислоты и избирательно сорбировать определенные размеры ДНК. В этой статье мы представляем простой протокол для очистки продуктов ПЦР с возможностью поглощения необходимых молекул ДНК. Было установлено, что использование оптимизированной буферной системы ПЭГ / NaCl с магнитным силикагелем в соотношении 1,5: 1 с продуктом ПЦР позволяет избавиться от фрагментов ДНК 100 и менее пар оснований (п.н.), а также от других загрязняющих веществ сохраняя более 90% ДНК в растворе. Соотношение 0,35: 1 обеспечивает высокоаффинную сорбцию молекул ДНК более 400 п.н. Практическое использование позволило улучить качество получаемых результатов секвенирования без увеличения стоимости исследований.

Ключвые слова: магнитные частицы, покрытые силикагелем, очистка, ДНК, ПЦР продукты.

ПТР ӨНІМДЕРІН СИЛИКЕГЕЛМЕН ҚАПТАЛҒАН МАГНИТТІ БӨЛШЕКТЕРМЕН ТАЗАЛАУ ӘДІСІН ОҢТАЙЛАНДЫРУ

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ТҮЙІН

Молекулалық – генетикалық зерттеулердегі маңызды мәселелердің бірі нуклеин қылшылдарын тазалау. Толық геномдық секвенирлеу технологиясының дамуы нуклеин қышқылдарының тазалығына деген талапты, сонымен қатар ДНҚ фрагментін өлшемі бойынша сұрыптау жүргізуді күшейтті. Құрамында ПЭГ/NaCl және магнитті түіршіктер бар буферлі жүйе нуклеин қышқылдарын тазартып, ДНҚ-ның белгілі

өлшемдерін сіңіруге мүмкіндік береді. Бұл мақалада ПТР өнімдерін, қажетті ДНҚ молекулаларын сіңіру арқылы тазартудың қарапайым әдісін ұсынамыз. ПЭГ / NaCl мен магнитті силикагелдің оңтайландырылған буферлі жүйені, ПТР өнімімен 1,5:1 ара-қатынаста қолдану арқылы, ДНҚ фрагменттерінің 100 ж.н және одан да кем жұп негіздерінен, басқа да ластанушылардан құтылуға мүмкіндік туғызады. Сонымен қатар ДНҚ-ның 90%-ы сақталады. 0,35:1 ара-қатынасты қолдану ДНҚ-ның 400 ж.н.-тен жоғары молекулаларды сіңіруді қамтамасыз етеді. Әдісті практикалық қолдану арқылы алынатын секвенирлеу нәтижелерін сапасын зерттеудің құнын жоғарлатпай жақсартуға үлесін тигізді .

Негізгі сөздер: силикагелмен қапталған магнитті бөлшектер, тазарту, ДНҚ, ПТР өнімдер.