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RIBONUCLEIC ACID ISOLATION FROM HUMAN MONONUCLEAR CELL CULTURE WITH MAGNETIC BEADS PRE-ENRICHMENT FOR MOLECULAR ANALYSIS

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Abstract: **Introduction**: In order to develop immunotherapies and therapeutic humoral molecules, ribonucleic acid (RNA) from cultured mononuclear cells (MNCs) is needed. However, it is not possible to isolate RNA using the standard mini column method from older MNC cultures. Therefore, the aim of this study was to develop a method to isolate RNA from MNC cultures, particularly older ones.

Materials and Methods: MNC cultures were grown from human buffy coats. The media from the cell culture was centrifuged to generate a pellet, to which CD45-specific magnetic beads were added. RNA was then isolated using the mini column method. The housekeeping gene beta-actin was used to confirm the success of RNA isolation through both real-time and conventional PCR tests.

Results: RNA was successfully isolated from MNC cultures, especially those that were a few months old, after pre-enrichment with magnetic beads. Without the magnetic bead pre-enrichment step, RNA isolation was not achieved. The results of the housekeeping gene tests indicated successful RNA isolation in all cases through both real-time and conventional PCR. Additionally, spectrophotometric values of the isolated RNA confirmed successful isolation.

Conclusion: This study is the first to demonstrate that it is possible to isolate RNA from human MNC cultures, particularly older ones, using specific magnetic beads. This method opens new opportunities for conducting genetic analyses, biomarker confirmation, and the development of antibodies.

Keywords: Mononuclear cells, RNA isolation, Mini column method, Polymerase chain reaction.

INTRODUCTION

Human mononuclear cell (MNC) cultures are generated for various purposes, such as gene therapies, immunotherapies, and antibody production. The applications of these cell cultures are increasing as the isolation of MNCs becomes feasible in many laboratories worldwide. There is a need to isolate nucleic acids from these cell cultures for different molecular analyses. These cultures can be successfully stored in various freezing media for later use (1-7).

Nucleic acid isolation is crucial for conducting various analyses, including the detection of pathogens, genetic mutations, and specific biomarkers. Different methods exist for nucleic acid isolation, such as the mini column and magnetic beads methods (8-12).

The difficulty arises when attempting to isolate nucleic acids, especially ribonucleic acid (RNA), from MNC cultures that are several months old. In our laboratory, we attempted to isolate nucleic acids from such MNC cultures using both the mini column and magnetic beads methods for over two years without success. It appears that mononuclear cells become resistant to nucleic acid isolation after being cultured for extended periods. Other research groups have also reported facing similar challenges (oral communications from different research groups and discussed elsewhere). The literature lacks protocols for isolating RNA in these cases.

Therefore, we decided to develop a method to isolate nucleic acids from MNC cultures older than four months. This method is based on magnetic beads pre-enrichment.

MATERIAL AND METHODS

Human buffy coats were provided by the Red Cross, Germany. They were supplied with numbers instead of names, ensuring donor anonymity and eliminating the need for ethics committee approval.

Isolation of MNCs: Isolation was performed using an MNC isolator. 500 µl of buffy coat was mixed with 4500 ml PBS (Biochrome, Germany). This mix-

ture was kept at room temperature for 10 minutes, then centrifuged and washed twice. The number of cells was counted using a Neubauer chamber under a microscope (13).

Culturing of MNCs: The MNCs were cultured in DMEM (Dulbecco's Modified Eagle Medium) and RPMI (Roswell Park Memorial Institute) solutions with 1% antibiotics (streptomycin, penicillin), 1% glutamine, and 2-3% FCS. Cultures were maintained in a CO_2 incubator at 37°C. All media components were sourced from Biochrome, Germany, or Lonza, USA (11). The cells were fed once a week, and the media was collected for various assays. Cell health was regularly monitored under a microscope, and cultures were maintained for over six months.

Isolation of Ribonucleic Acid: For RNA isolation, 5 ml of media containing MNCs was centrifuged to generate a pellet. Pellets were also generated from cultures using trypsin. This pellet was diluted in 100 µl of PBS and used to isolate RNA with a mini column kit (Genekam, Germany). This method, referred to as Method 1, failed consistently over two years (12).

Development of a New Method Using Magnetic Beads: In the new method, 10 to 20 µl of CD45 magnetic beads (Genekam) were added to the diluted pellet and incubated at room temperature for 15 minutes in a 2 ml microtube. Then, 1.5 ml PBS was added, and the microtube was placed in a magnetic rack (Genekam). Cells attached to the magnetic beads were attracted to the magnets, forming a pellet. The supernatant was discarded, and the pellet was washed twice with PBS in the magnetic rack. The collected pellet with magnetic beads was resuspended in 50 µl of PBS or used directly for nucleic acid isolation. Alternatively, magnetic beads were added directly to 3 ml of media containing cells and incubated at room temperature for 15 minutes. The microtubes containing bead-bound cells were placed in a magnetic rack to generate a pellet (1.5 ml media was pipetted into two microtubes for use in the magnetic rack) and washed as described above (Figure 1).

Nucleic Acid Isolation: RNA isolation was performed using a mini column isolation kit (Genekam, Germany). Briefly, 20 µl of the suspended pellet was added to lysis buffer 1 (Tube A) with proteinase K and incubated at 56°C for 15 minutes. Lysis buffer 2 and molecular ethanol were then added. This solution was passed through a mini column and washed twice. Finally, RNA was eluted in 50 to 100 µl of elution buffer and stored at -20°C for further use. The complete isolation protocol is available from the manufacturer (12).

Confirmation of Successful RNA Isolation: The success of RNA isolation was confirmed using a spectrometric instrument, Nanodrop (ThermoFisher, USA), and spectrophotometric values were measured.

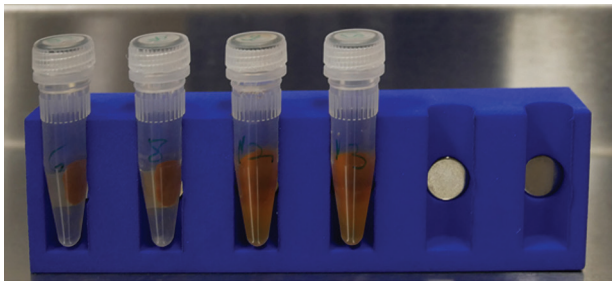

Figure 1. *Magnetic rack with microtubes containing magnetic beads with MNC*

The machine was calibrated with elution buffer, and 2 µl of isolated RNA per isolation was used to measure the yield. The yield per µl was recorded.

PCR Assays: Confirmation of successful RNA isolation was performed using conventional and real-time PCR assays for the human internal control beta-actin. For conventional PCR, 2 µl of RNA in a total volume of 20 µl was used with a PCR kit (Genekam) in a thermocycler (Biometra, Germany), and the results were visualized as bands in 2% agarose gel. For real-time PCR, 2 µl of RNA in a total volume of 20 µl was used in a real-time PCR assay on a thermocycler (ABI 7500, ThermoFisher, USA), and results were observed as smooth curves and Ct values. Positive and negative controls were used. Full protocols for these assays are available from the manufacturers.

Further assays were performed after converting RNA into cDNA, but these will be detailed in future publications.

RESULTS

There was no RNA isolation without using magnetic beads for two years with Method 1. After introducing CD45-specific magnetic beads, successful RNA isolations were achieved in all samples, as shown in Table 1. The results of 15 isolations are presented as examples. In total, over 100 such isolations were performed successfully over three years, confirmed through spectrometric values. Further confirmation was obtained through conventional PCR and real-time PCR for the human housekeeping gene beta-actin (Figure 2), with Ct values ranging between 17 and 22.

The isolated volume was 100 µl per sample, sufficient for multiple molecular analyses. Using 20 µl of magnetic beads was adequate to isolate RNA from MNC cultures, even those older than six months. The method was also successful with fresh MNC cultures (a few days old).

Magnetic bead isolation using CD45-specific magnetic beads was quick, taking less than 30 minutes. The quality of the magnetic rack was crucial; the rack used in this work was designed to fit microtubes

Table 1. *Results of RNA isolation from MNC cultures with and without magnetic beads with different parameters*

Experiment	RNA isolations without magnetic beads	RNA isolations with magnetic beads	Real time PCR (beta actin)	cDNA synthesis	Spectrometric measurement
1	failure	–	–	–	–
2	failure	–	–	–	–
3	failure	–	–	–	–
4		successful	+	+	+
5		successful	+	+	+
6		successful	+	+	+
7		successful	+	+	+
8		successful	+	+	+
9		successful	+	+	+
10		successful	+	+	+
11		successful	+	+	+
12		successful	+	+	+
13		successful	+	+	+
14		successful	+	+	+
15		successful	+	+	+

tightly, allowing for easy disposal of the supernatant by tilting the rack into a waste container. This method worked with both pellets and culture media, where magnetic beads were added directly into MNC media without generating a pellet.

These isolations were successfully converted into cDNA and used for further applications, which will be detailed in future publications.

DISCUSSION

In this research, we developed a method to isolate RNA from MNC cultures. Many laboratories working with MNC cultures face the challenge of isolating RNA from these cells. We encountered significant difficulties isolating nucleic acids from our cell cultures, and our sales representatives frequently received the same questions during visits to various universities in Germany. This motivated us to solve the problem.

The method presented here uses magnetic beads to isolate MNCs, which are then used to isolate nucleic acids with the mini column method. This method yields a sufficient quantity of nucleic acid (100 µl per sample) for various molecular analyses. In comparison, direct isolation without magnetic beads was unsuccessful, as PCR tests of such isolations failed to produce any signals.

The magnetic bead isolation method works well with both pellets and cell culture media containing MNCs. This step is completed within 30 minutes, and

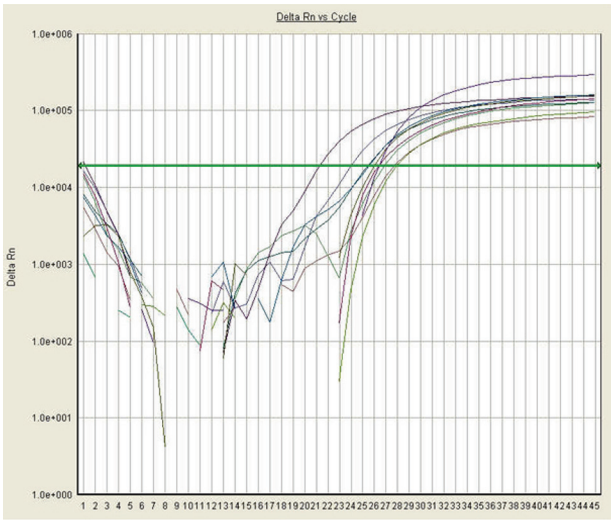

Figure 2*. The result for successful presence of isolated RNA from MNC cultures with real time PCR*

RNA isolation with the mini column takes an additional 35 minutes, resulting in a total isolation time of about an hour. The results were comparable to those obtained from isolations of different human tissue samples in our other research (14).

In this study, we used 2-3% FCS, consistent with existing literature, thus reaffirming that 2-3% FCS can be effectively used in cell culture, saving costs and improving animal rights (11).

Other groups adopting this method should consider the quality of magnetic racks, as some available on

the market have drawbacks, such as microtubes shaking during the process, leading to the need for many pipette tips. In our case, the microtubes fit well in the magnetic rack, allowing for quick disposal of the supernatant by tilting the rack, which makes the process faster (Figure 1).

Developing this method took us two years and involved numerous experiments, though we have presented only a few in our results.

CONCLUSION

This work may be the first to report the development of a robust method for isolating RNA from human MNC cultures, particularly those a few months old, using a magnetic beads pre-enrichment step followed by mini column isolation. The nucleic acid isolated can be used for various molecular applications, including the analysis of genetic targets, isolation of protein-specific genes, and creating the basis for generating new types of antibodies for therapeutic and diagnostic use.

Abbreviations

Ct - Threshold Cycle
DMEM - Dulbecco's Modified Eagle Medium
FCS - Fetal Calf Serum
MNC - Mononuclear Cells
PCR - Polymerase Chain Reaction
RNA - Ribonucleic Acid
RPMI - Roswell Park Memorial Institute

Funding: There was no external funding.

Conflicts of Interest: There are no conflicts of interest.

Note: No artificial intelligence tools were used in these studies.

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Sažetak

IZOLACIJA RIBONUKLEINSKE KISELINE (RNA) IZ LJUDSKIH MONONUKLEARNIH ĆELIJA SA PRETHODNIM OBOGAĆIVANJEM MAGNETNIM PERLAMA ZA MOLEKULARNU ANALIZU

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Uvod: Za razvoj imunoterapija i terapijskih antitela, neophodna je ribonukleinska kiselina (RNA) iz kultivisanih mononuklearnih ćelija (MNC). Međutim, nije moguće izolovati RNA standardnom metodom mini kolona iz starijih MNC kultura. Stoga je cilj ovog istraživanja bio razviti metod za izolaciju RNA iz MNC kultura, posebno starijih.

Materijali i metode: MNC kulture su bile uzgajane iz humanih „buffy coats". Medijum iz ćelijske kulture je centrifugiran kako bi se generisao pelet, a kome su dodate CD45-specifične magnetne perle. RNA je zatim izolovana koristeći metodu mini kolona. Housekeeping gen beta-aktin korišćen je kako bi se potvrdio uspeh izolacije RNA putem realno-vremenskih i konvencionalnih PCR testova.

Rezultati: Izolacija ribonukleinske kiseline (RNA) iz MNC kultura je postignuta uspešno, posebno kod onih koje su bile nekoliko meseci stare, nakon predobogaćivanja magnetnim perlama. Bez koraka predobogaćivanja magnetnim perlama, izolacija RNA nije bila ostvariva. Rezultati testova housekeeping gena su pokazali uspešnu izolaciju RNA u svim slučajevima putem realno-vremenskih i konvencionalnih PCR metoda. Dodatno, spektrofotometrijske vrednosti izolovane RNA su potvrdile uspešnu izolaciju.

Zaključak: Ova studija prva pokazuje da je moguće izolovati RNA iz ljudskih MNC kultura, posebno starijih, korišćenjem specifičnih magnetnih perli. Ovaj metod otvara nove mogućnosti za sprovođenje genetičkih analiza, potvrdu biomarkera i razvoj antitela.

***Ključne reči**:* Mononuklearne ćelije, Izolacija RNA, Metoda mini kolona, Lančana reakcija polimeraze.

REFERENCES

1. Seeger FH, Tonn T, Krzossok N, Zeiher AM, Dimmeler S. Cell isolation procedures matter: a comparison of different isolation protocols of bone marrow mononuclear cells used for cell therapy in patients with acute myocardial infarction. Eur Heart J. 2007; 28(6): 766–72. doi: 10.1093/eurheartj/ehl509.

2. Lu RM, Hwang YC, Liu IJ, Lee CC, Tsai HZ, Li HJ, et al. Development of therapeutic antibodies for the treatment

of diseases. J Biomed Sci. 2020; 27(1): 1. doi: 10.1186/s12929-019-0592-z.

3. Heeschen C, Lehmann R, Honold J, Assmus B, Aicher A, Walter DH, et al. Profoundly reduced neovascularization capacity of bone marrow mononuclear cells derived from patients with chronic ischemic heart disease. Circulation. 2004; 109(13): 1615-22. doi: 10.1161/01.CIR.0000124476.32871.E3.

4. Hombach A, Hombach AA, Abken H. Adoptive immunotherapy with genetically engineered T cells: modification of the IgG1 Fc 'spacer' domain in the extracellular moiety of chimeric antigen receptors avoids 'off-target' activation and unintended initiation of an innate immune response. Gene Ther. 2010; 17(10): 1206-13. doi: 10.1038/gt.2010.91.

5. Levine BL, Miskin J, Wonnacott K, Keir C. Global Manufacturing of CAR T Cell Therapy. Mol Ther Methods Clin Dev. 2016; 4: 92-101. doi: 10.1016/j.omtm.2016.12.006.

6. Fibriansah G, Lok SM. The development of therapeutic antibodies against dengue virus. Antiviral Res. 2016; 128: 7-19. doi: 10.1016/j.antiviral.2016.01.002.

7. Wolf C, Köppert S, Becza N, Kuerten S, Kirchenbaum GA, Lehmann PV. Antibody levels poorly reflect on the frequency of memory B cells generated following SARS-CoV-2, seasonal Influenza, or EBV Infection. Cells. 2022; 11(22): 3662. doi: 10.3390/cells11223662.

8. Lee KO, Park MY, Kim LH, Seong HS, Park BH, Jeong SJ. Pandemic novel influenza a (H1N1) virus in Korea: the experience from august to september 2009. Korean J Clin Lab Sci. 2009; 41: 145-52.

9. Muller MG, George AR, Walochnik J. Acinetobacter baumannii in localised Cutaneous Mycobacteriosis in Falcons. Vet Med Int 2010; 2010: 321797. doi: 10.4061/2010/321797.

10. Bhatia S. Pitfalls found in SARS CoV-2 specific test performance during the comparison between WHO recommended method and a commercial test. Atlantic J Med Sci Res 2023; 3(1): 22-6. doi:10.5455/atjmed.2022.12.024.

11. Boom R, Sol CJ, Salimans MM, Jansen CL, Wertheim-van Dillen PM, van der Noordaa J. Rapid and simple method for purification of nucleic acids. J Clin Microbiol. 1990; 28(3): 495-503. doi: 10.1128/jcm.28.3.495-503.1990.

12. Bhatia S. 2-3% fetal calf serum in cell media is sufficient for the development and growth of human mononuclear cell cultures. Atlantic J Med Sci Res. 2023; 3(2): 46-9. doi: 10.5455/atjmed.2023.01.06.

13. Bhatia S, Baersch G. Rapid isolation of mononuclear cells with high yield from minimal blood volumes: a simplified and robust approach for immunotherapeutic applications. Med Sci Discov. 2023; 10(10): 838–41. doi: 10.36472/msd.v10i10.1062.

14. Bhatia S. Loss of an abundant quantity of ribonucleid acid during mini column isolation method. Biotechnologia Acta. 2023; 16(3): 65-9. doi: 10.15407/biotech16.03.065

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