Journal of the Serbian Chemical Society
JSCS-info@shd.org.rs • www.shd.org.rs/JSCS
J. Serb. Chem. Soc. *88* (7–8) 729–738 (2023)
JSCS–5658
Original scientific paper
Published 6 August 2023

Post-TRIzol protein extraction from peripheral blood mononuclear cells

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(Received 30 March, revised 27 April, accepted 8 July 2023)

Abstract: After sample processing for RNA and DNA analysis, the leftover protein pellets are usually discarded due to the limited efficiency of pellet reconstitution/solubilisation. As the pelleted proteins are tightly packed, they are most often solubilised using chaotropic agents (*e.g.*, guanidine hydrochloride or urea), detergents (*e.g.*, SDS), salts (NaCl) or basic buffer (Tris). The aim of this study was to define and optimise the procedure for the efficient extraction of proteins from human peripheral blood mononuclear cells (PBMCs), obtained by a single blood draw and lysed in TRIzol reagent, by varying experimental conditions in terms of protein precipitation solvent (isopropanol or acetone), washing (with or without guanidine hydrochloride) and solubilisation solution (containing SDS, NaCl, urea and/or Tris). We evaluated the efficacy of the final, optimised protocol to solubilise both small cytoplasmic and larger transmembrane proteins, and the compatibility with methods employed for the subsequent analysis of protein posttranslational modifications, such as glycosylation. The optimised protocol for the extraction and isolation of post-TRIzol leftover proteins from PBMCs can be defined as follows: protein precipitation from the organic phase with ice-cold acetone, pellet washing with absolute ethanol and solubilisation in 1 % SDS, employing 20 min heating at 50 °C and vortexing.

Keywords: protein solubilisation; TRIzol extraction; PBMC.

INTRODUCTION

Extraction of RNA, DNA and proteins from the same sample enables the exploration of cellular mechanisms at different levels of gene expression. A comprehensive evaluation of RNA-based and protein-based changes related to physiological and pathological stimuli is crucial for understanding the molecular basis

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https://doi.org/10.2298/JSC230330037S

of cellular processes, due to the dynamics and the complexity of gene regulation. Simultaneous extraction of different types of biomolecules becomes extremely important when experimental samples are hard to obtain and/or process, or when the number of harvested cells or tissue size per sampling is relatively small.[1]

Since leucocyte counts and alterations in their signalling pathways and gene expression pattern reflect the immunological status, peripheral blood mononuclear cells (PBMCs) and cultured lymphocytes are frequently used as biological material in immunological studies, cancer and diabetes research, as well as in pathogen-host interaction examinations.[2] In many of these experimental settings, the comprehensive assessment of changes in different biomolecules and their correlations is biologically relevant and crucial for the interpretation of results. Therefore, a method for rapid, efficient and reproducible simultaneous extraction of RNA and proteins from PBMCs or their cellular fractions is much needed, as the sample size and the number of individual sample acquisitions is limited.

The widely applied method for this simultaneous extraction of all mentioned biomolecules includes the usage of phenol, guanidine isothiocyanate and isoamyl alcohol mixture, commercially branded as TRIzol. Procedures which employ TRIzol are mostly optimised for RNA extraction, while the leftover organic phase, separated after the addition of chloroform, can be used for the isolation of DNA and proteins from the same sample, which is more cost-effective than the purchase of specialised commercially available kits for the simultaneous extraction of different biomolecules.[1] Most post-TRIzol protein-leftover processing methods include the employment of guanidine hydrochloride, urea and SDS, in specific concentrations, in order to solubilise a protein precipitate.[3,4] Solubilisation efficiency depends on the composition of the solubilisation buffer and temperature regime, as well as on the pellet size and the initial protein composition of the sample.[4,5] Therefore, the optimal protocol for the protein extraction from TRIzol lysates is expected to vary between different tissue/cell types.

The aim of the present study was to define and optimise the procedure for the efficient protein extraction from human PBMCs, obtained by a single blood draw and lysed in TRIzol reagent, by varying experimental conditions in a several-step process. In an effort to maximise the protein yield, limit the utilisation of strong chaotropic agents, avoid dialysis and shorten the total duration of the extraction procedure, we tested alternative organic solvents for protein precipitation, different pellet washing procedures, as well as several alternative solubilisation solutions/buffers, in terms of NaCl, SDS, urea and Tris concentration. We evaluated the efficacy of the optimised protocol for the solubilisation of small soluble cytoplasmic and larger transmembrane proteins, as well as the compatibility with the subsequent analysis of protein posttranslational modifications, such as glycosylation.

EXPERIMENTAL

PBMC isolation and lysis

Peripheral blood samples were collected from healthy adult volunteers (n = 16) in EDTA-containing tubes after an overnight fasting. Samples were pooled and centrifuged at 400*g* (deceleration brakes off) at 4 °C for 10 min in a centrifuge with the swing-bucket rotor (centrifuge 5804R, Eppendorf, Hamburg, Germany). The buffy coat was collected, diluted with phosphate-buffered saline (PBS, 10 mM, pH 7.4) in a 1:2 volume ratio, and layered onto lymphocyte separation medium (1.077 g/mL, Capricorn Scientific, Ebsdorfergrund, Germany). The manufacturer's protocol was modified for the isolation of PBMCs, by including centrifugation during the separation step at 700*g* (slow acceleration, deceleration brakes off, swing-bucket rotor) at 18–21 °C for 30 min (centrifuge 5804R, Eppendorf, Hamburg, Germany). PBMC layer was collected, washed twice with PBS and once with erythrocyte lysis buffer (155 mM NH_4Cl, 10 mM $KHCO_3$, 0.1 mM EDTA), and centrifuged at 100*g* at room temperature for 10 min. PBMC pellet was dissolved in TRIzol reagent (TRIsure, Bioline, London, UK), assuring complete solubilisation and homogenization, and aliquots stored at –80 °C for further processing, which is a standard requirement of sample storage for RNA extraction, necessary for preserving the integrity of RNA molecules. The amount of TRIzol reagent used for the lysis of PBMCs extracted from the initial 3 mL (*ca*. 2.5×10^6 cells) of whole peripheral blood was 400 μL.

Protein extraction and solubilisation

As shown in Fig. 1, thawed PBMC lysates in TRIzol reagent were subjected to phase separation according to the manufacturer's instruction,[6] by adding chloroform (0.2 mL per 1 mL of TRIzol reagent) and centrifugation at 12000*g* at 4 °C for 15 min. The aqueous phase was removed and absolute ethanol added (0.3 mL per 1 mL of TRIzol reagent) to the remaining interphase and phenol–chloroform organic phase in order to precipitate DNA. Samples were mixed by gentle inversion and DNA pelleted by centrifugation at 2000*g*, at 4 °C for 5 min. The remaining supernatants (phenol–ethanol solutions) were pooled together and separated into equal aliquots in 1.5 mL microtubes for further protein precipitation, washing and solubilisation following different protocols.

Either ice-cold isopropanol or ice-cold acetone (1.5 mL per 1 mL of TRIzol reagent used for PBMC homogenization) was added to phenol–ethanol supernatant to precipitate proteins and incubated at –20 °C for 30 min. The centrifugation step at 12000*g*, at 4 °C for 10 min was performed to pellet proteins. Pellets were washed twice following two alternative protocols: using 0.3 M guanidine hydrochloride (Gu-HCl) in 95% ethanol or absolute ethanol (2 mL per 1 mL of TRIzol reagent), at 4 °C for 20 min and centrifugation at 7500*g* at 4 °C for 5 min. For pellets washed with Gu-HCl, a third washing step was performed with absolute ethanol under the same conditions. Protein pellets were air-dried and resuspended in 100 μL of the tested solubilisation solutions/buffers:

Solut. 1 – 1% SDS in ddH_2O;

Solut. 2 – 100 mM Tris pH 8.0, 5 mM EDTA, 1 % SDS;

Solut. 3 – 100 mM Tris pH 8.0, 5 mM EDTA, 140 mM NaCl, 1 % SDS;

Solut. 4 – 100 mM Tris pH 8.0, 5 mM EDTA, 6 M urea, 5 % SDS.

Each mixture was vortexed and incubated in a thermoshaker at 50 °C and 300 rpm to enable protein solubilisation, for varying incubation times (10–40 min) and with intermittent vortexing, and finally left at 90 °C for 10 min. After cooling down for 2–3 min, centrifuging at 10000*g* for 10 min was conducted to remove any remaining insoluble aggregates.

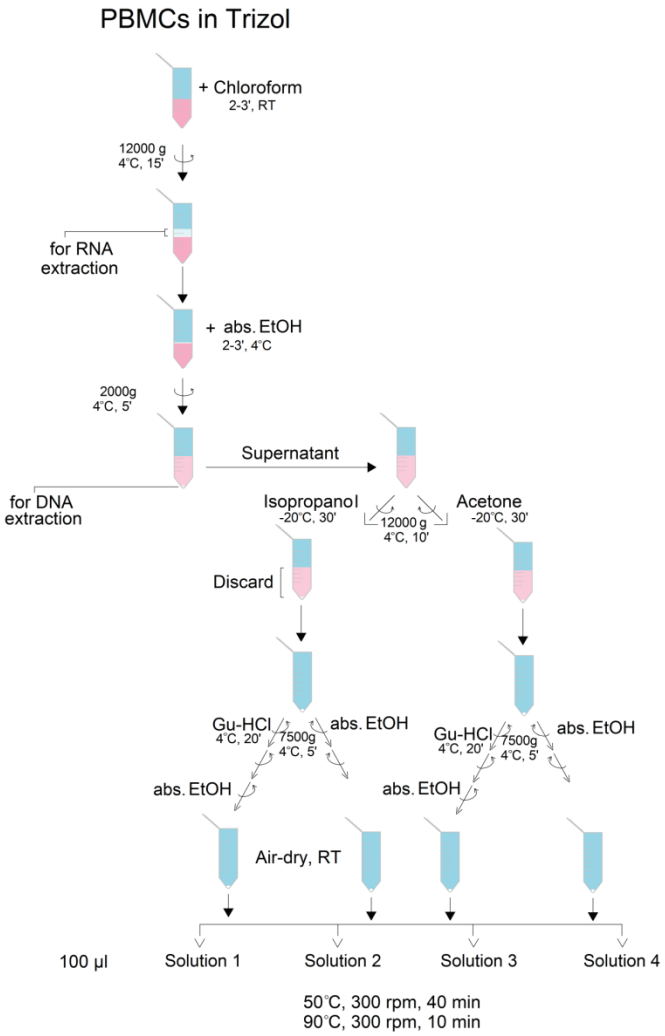

Fig. 1. Schematic presentation of the experimental setup.

In a separate experimental setup, we tested the effect of selected organic solvents used for protein precipitation on the time-dependency of the pellet solubilisation. Protein pellets were solubilised in solution 1, vortexed and incubated at 50 °C and 300 rpm for 40 min, and then heated at 90 °C for 10 min with continuous shaking. Every 10 min, samples were cooled down for 2–3 min to the room temperature, 2 µL aliquots were taken to measure the absorbance at A280 (Epoch microplate spectrophotometer, BioTek Instruments – Agilent, Santa Clara, CA, USA), and 10 µL aliquots were saved for electrophoresis.

Electrophoretic separation

Sample aliquots were mixed in 1:1 volume ratio with 2× concentrated Laemmli buffer containing 2-mercaptoethanol as a reducing agent (100 mM Tris-HCl, pH 6.8, 4 % SDS, 20 %

glycerol, 10 % 2-mercaptoethanol, 0.1 % bromophenol blue) and heated at 95 °C for 5 min. Samples were centrifuged prior to SDS-polyacrylamide gel electrophoresis (SDS-PAGE) to remove possible aggregates. SDS-PAGE was used for the electrophoretic separation of proteins on 10 or 8 % gels under reducing conditions. Ten percent gels were used to examine potential presence of aggregates, whereas 8 % gels were employed for separation of higher molecular weight proteins, further enabling their immunodetection. Samples (concentration 2 mg/mL) were applied at different volumes (10, 15 or 30 μL). The molecular weight of proteins was estimated using Pink Plus prestained protein ladder with a 14–175 kDa range (Cleaver Scientific, Rugby, UK). Following electrophoretic separation, proteins were either stained on gel or transferred to a nitrocellulose membrane. In-gel protein staining was performed using the standard procedure with Coomassie brilliant blue R250 (CBB) solution.[7] Selected lectins and specific antibodies were employed for detection of (glyco)proteins.

Lectin- and immuno-blotting

Following protein transfer onto nitrocellulose membrane (wet transfer: constant voltage, 100 V for 1 h, with cooling unit, as recommended by the producer), protein-free sites were blocked with 5 % BSA in Tris-buffered saline (10 mM Tris, 0.15 M NaCl, pH 7.4) containing 0.1 % Tween-20 (TBST) overnight (for lectin blotting) or for 45 min (for immunoblotting). Membranes were washed in TBST before immunodetection. For lectin blotting, membranes were incubated in a solution of biotinylated *Sambucus nigra* lectin (SNA) or *Aleuria aurantia* lectin (AAL) in TBST (0.2 μg/mL) at room temperature for 1 h, washed with TBST 4×5 min, further incubated with fluorescein-conjugated streptavidin (0.025 μg/mL) at room temperature for 1 h and washed again with TBST 4×5 min. The fluorescent signal was recorded using the ChemiDoc MP imaging system (Bio-Rad Laboratories Inc., Hercules, CA, USA). SNA, AAL and fluorescein-conjugated streptavidin were purchased from Vector Laboratories (Burlingame, CA, USA). For immunodetection, membranes were incubated in a solution of primary antibodies in TBST, at 4 °C overnight, washed with TBST 6×5 min and further incubated with biotinylated secondary antibodies (0.15 μg/mL) at room temperature for 30 min. Another washing step was applied (6×5 min), and the membranes incubated with fluorescein-conjugated streptavidin as previously described. The following primary antibodies were employed: mouse monoclonal anti-CD45 antibody (1 μg/mL; Bio-Rad, Hercules, CA, USA), rabbit anti-*β*-actin antiserum (1:500 volume ratio; Sigma Aldrich) and goat polyclonal anti-GAPDH antibody (0.625 μg/mL; Sigma Aldrich). Biotinylated secondary antibodies were: horse-anti-mouse, horse-anti-goat, and goat-anti-rabbit IgG (Vector Laboratories, Burlingame, CA, USA).

RESULTS AND DISCUSSION

The aim of our study was to define and optimise the procedure for the efficient protein extraction from human PBMC. Following the manufacturer's procedure for TRIzol application, proteins from PBMCs precipitated with isopropanol and washed with Gu-HCl and ethanol were completely dissolved in all examined solutions under the defined conditions. Our selection of the solubilisation solutions was based on the previous attempts to define procedures for the efficient extraction of proteins from TRIzol lysates of other biological samples. As the pelleted proteins are tightly packed, they are usually solubilised using some chaotropic agents (*e.g.*, guanidine hydrochloride or urea), detergent (*e.g.*, SDS) or salts (NaCl, Tris). It is recommended to avoid chemicals that are harm-

ful and toxic, as well as those that can interact with proteins resulting in misleading data and conclusions. Profile of protein bands as well as their intensities were similar in all tested conditions, as shown in Fig. 2A. This result differs from those reported by others,[8–13] who have found the increased efficiency of the solutions containing (thio)urea compared to alternative solubilisation solutions for proteins isolated by TRIzol method from tissues and cultured cell other than PBMCs. Urea is a non-toxic, strong protein denaturant that facilitates protein solubilisation in combination with disulphide bond disruption.[14] Still, the side effect of urea employment is carbamylation, a process in which urea dissociates to ammonia and cyanate with concomitant formation of isocyanic acid. The acid further interacts with the N-termini of proteins and amino groups of lysine and arginine. Approximatelly 20 % of N-termini and 2 % of lysine residues are modified with as much as 2 M urea.[15] Carbamylation interferes with downstream processes, *e.g.*, enzymatic digestion and peptide separation, identification and quantification, as well as protein labelling. Therefore, we excluded urea from further experiments.

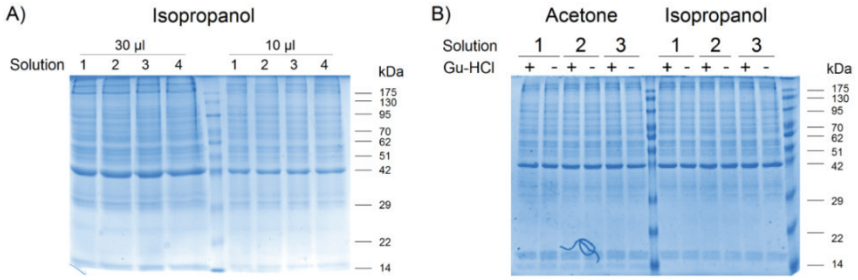

Fig. 2. SDS-PAGE electrophoresis on 10 % gel under reducing conditions. A) Proteins precipitated with isopropanol, washed with the addition of guanidine hydrochloride (Gu-HCl) and solubilised in one of the four tested solutions; B) proteins precipitated with isopropanol or acetone, washed with or without the addition of Gu-HCl. Solutions used for solubilisation are labelled as follows: 1 (1 % SDS), 2 (100 mM Tris, pH 8.0, 5 mM EDTA, 1 % SDS), 3 (100 mM Tris, pH 8.0, 5 mM EDTA, 1 % SDS, 140 mM NaCl) and 4 (100 mM Tris, pH 8.0, 5 mM EDTA, 5 % SDS, 6 M urea). Molecular weight markers are given on the right.

None of the variables examined, in respect to the type of the organic solvent and the composition of the washing or solubilisation solution, demonstrated deviations from other experimental combinations regarding the yield of the extracted proteins or the distribution of proteins after SDS-PAGE (Fig. 2B). Acetone was equally suitable for protein precipitation as the recommended isopropanol. The exclusion of Gu-HCl from the pellet washing step did not affect the protein yield and the solubilisation efficiency. Our finding is in accordance with the results of Kopec *et al.*[4] who analysed the efficacy of protein extraction from brain tissue by varying concentrations of NaCl, Tris, SDS and EDTA in the lysis buffer. Both

results confirmed that guanidine hydrochloride is not a crucial component in the washing step, thus, the usage of this highly toxic chaotropic agent can be omitted. According to our results, any of the tested solubilisation solutions could be used for protein solubilisation giving similar protein yields (Fig. 2B). Therefore, we selected the simplest solution (1 % SDS in ddH_2O) for the protein solubilisation step. SDS, a strong anionic detergent, is widely employed protein denaturing agent and is often incorporated in lysis/solubilisation buffers. Furthermore, SDS is more compatible with some sophisticated techniques (such as HPLC and FPLC) and, hence, more suitable than urea. SDS will not crystalize under often-used conditions (1 % or 34 mM), unlike urea whose concentrations are much higher (6–8 M) and crystallization is inevitable.[16]

It was noticed that proteins in acetone-precipitated pellet were less tightly packed and aggregates tended to dissolve faster than those precipitated with isopropanol. Since all washing and solubilisation procedures resulted in the same outcome, to examine the efficiency of the solubilisation with time, the absorbance of dissolved proteins was measured in 10-min intervals during 40 min, employing heating at 50 °C, plus the additional 10 min heating at 90 °C (termed a 50--min time point). The simplest method employing absolute ethanol for pellet washing and 1 % SDS for solubilisation was used to dissolve acetone- or isopropanol-precipitated proteins. Acetone pellet was dissolved more efficiently at 50 °C, while heating at 90 °C had no additional effect (Fig. 3).

All tested procedures resulted in comparable specific protein bands corresponding to small soluble proteins GAPDH (37 kDa) and β-actin (42 kDa), as well as high molecular mass transmembrane protein CD45 (~180–~220 kDa), as shown in Fig. 4A.

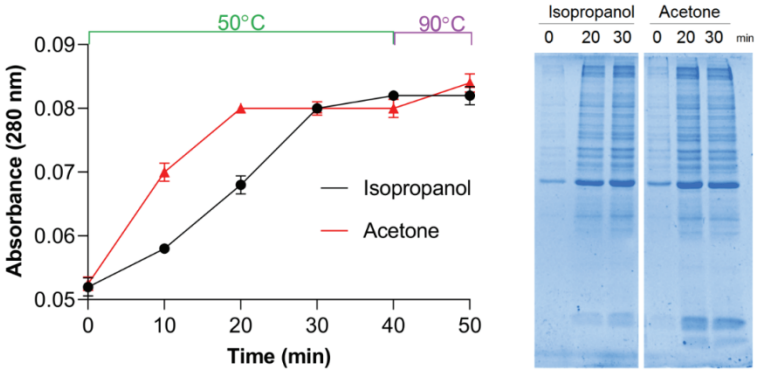

Fig. 3. Solubilisation curve of the protein pellet precipitated with isopropanol or acetone and CBB staining of the solubilised proteins precipitated with isopropanol or acetone (washed with absolute ethanol, dissolved in 1 % SDS) after electrophoretic separation (SDS-PAGE under reducing conditions) on 10 % gels. Sample aliquots were collected before incubation at 50 °C and every 10 min during 40 min incubation, and additional 10 min incubation at 90 °C.

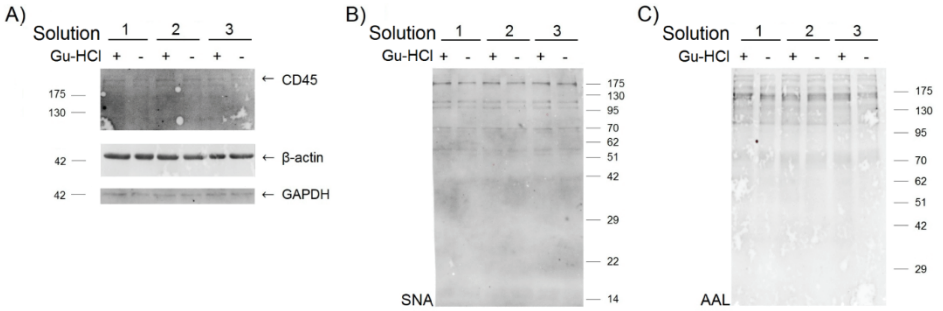

Fig. 4. Proteins precipitated with acetone, washed with ethanol and dissolved in 1 % SDS resolved by reducing SDS-PAGE on 10 % gel (for GAPDH, *β*-actin and SNA-reactive glycoproteins) or 8 % gel (for CD45 and AAL-reactive glycoproteins). Immunoblot with anti-CD45, anti-*β*-actin or anti-GAPDH antibodies (A) and lectin blots with SNA (B) or AAL lectin (C).

In order to detect whether posttranslational modifications of proteins remained preserved during the isolation procedure, the presence of sialylated (recognised by SNA lectin) and fucosylated (recognized by AAL lectin) glycoforms in solubilisates obtained after acetone precipitation was examined and confirmed by lectin blotting. As can be seen from Fig. 4B and C, neither sialylation nor fucosylation pattern of the extracted proteins was affected by the presence/absence of Gu-HCl in the pellet washing step or by the choice of the tested solubilisation solutions. The distribution of the bands in the lectin blot and their intensities were similar regardless of the protein extraction procedure, both in the region of small and high molecular weight glycoproteins.

CONCLUSION

By varying solutions and conditions for protein precipitation, pellet washing and protein solubilisation, the optimised protocol for the extraction and isolation of post-TRIzol leftover proteins from PBMCs can be defined as follows: protein precipitation from the organic phase with ice-cold acetone, pellet washing with absolute ethanol and solubilisation in 1 % SDS employing 20 min heating at 50 °C and vortexing. The addition of urea, NaCl and Tris, as well as an increase in the concentration of SDS did not improve the solubilisation of PBMC protein pellet. Furthermore, the exclusion of Gu-HCl from the pellet washing solution did not affect the quality/quantity of the extracted proteins. Acetone was shown to be equally suitable for the protein precipitation as recommended isopropanol. Even more, acetone pellet was more easily solubilised during shorter periods of incubation (up to 20 min). The proposed protocol is specifically tailored for PBMCs, as a frequently analysed type of biological sample for biomedical and biochemical research. It may be considered as a simple, cost-effective and efficient protocol to extract PBMC proteins from post-TRIzol leftover material.

Acknowledgements. This work was supported by the Ministry of Science, Technological Development and Innovations (No 451-03-47/2023-01/200019).

ИЗВОД

ЈЕДНОСТАВНА МЕТОДА ИЗОЛОВАЊА ПРОТЕИНА БЕЗ УПОТРЕБЕ ГУАНИДИН--ХИДРОХЛОРИДА ИЗ ОСТАТАКА ЕКСТРАКЦИЈЕ РЕАГЕНСОМ TRIZOL ИЗ ПЕРИФЕРНИХ МОНОНУКЛЕАРНИХ ЋЕЛИЈА

ЈОВАНА СТЕВАНОВИЋ, ДРАГАНА РОБАЈАЦ, ОЛГИЦА НЕДИЋ и ЗОРАНА ДОБРИЈЕВИЋ

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Након екстракције RNA и DNA, преостали протеински остатак најчешће бива одстрањен услед његовог отежаног растварања. Исталожени протеини су густо паковани, због чега је најчешће потребно користити неки хаотропни агенс (нпр. гуанидин-хидрохлорид или уреа), детергент (нпр. SDS), со (NaCl) или базни пуфер (Tris). Циљ студије био је да се дефинише и оптимизује поступак ефикасне екстракције протеина из хуманих мононуклеарних ћелија периферне крви (PBMC), добијених из једног узимања крви и лизираних у реагенсу TRIzol. Варирани су експериментални услови у смислу избора растварача за таложење протеина (изопропанол или ацетон), испирања гуанидин-хидрохлоридом или без испирања и раствора за солубилизацију (садржај SDS, NaCl, урее и/или Tris). Процењена је ефикасност оптимизованог протокола за солубилизацију малих солубилних протеина цитоплазме и већих трансмембранских протеина, као и компатибилност методе са даљом анализом пост-транслационих модификација протеина (нпр. гликозиловања). Протокол оптимизован за екстракцију и изоловање протеина из остатака TRIzol лизата PBMC, дефинисан је као: преципитација протеина из органске фазе ледено-хладним ацетоном, испирање талога апсолутним етанолом и солубилизација протеина у 1 % SDS грејањем на 50 °C уз мешање, у трајању од 20 min.

(Примљено 30. марта, ревидирано 27. априла, прихваћено 8. јула 2023)

REFERENCES

1. W. Mathieson, G. A Thomas, *Anal. Biochem.* **433** (2013) 10 (https://dx.doi.org/10.1016/j.ab.2012.10.006)
2. P. Sen, E. Kemppainen, M. Orešič, *Front. Mol. Biosci.* **4** (2018) (https://dx.doi.org/10.3389/fmolb.2017.00096)
3. H. Riol, B. Jeune, A. Moskovic, L. Bathum, E. Wang, *Anal. Biochem.* **275** (1999) 192 (https://dx.doi.org/10.1006/abio.1999.4328)
4. A. M. Kopec, P. D. Rivera, M. J. Lacagnina, R. Hanamsagar, S. D. Bilbo, *J. Neurosci. Methods* **280** (2017) 64 (https://dx.doi.org/10.1016/j.jneumeth.2017.02.002)
5. R. B. Braakman, K. Bezstarosti, A. M. Sieuwerts, V. de Weerd, A. M. van Galen, C. Stingl, T. M. Luider, M. A. Timmermans, M. Smid, J. W. Martens, J. A. Foekens, J. A. Demmers, A. Umar, *J. Proteome Res.* **14** (2015) 1627 (https://dx.doi.org/10.1021/acs.jproteome.5b00046)
6. TRIsure™ Product Specifications, https://www.bioline.com/mwdownloads/download/link/id/953/trisure_product_manual.pdf (accessed 25 July, 2023)
7. C. Arndt, S. Koristka, A. Feldmann, R. Bergmann, M. Bachmann, *Methods Mol. Biol.* **1853** (2018) 27 (https://dx.doi.org/10.1007/978-1-4939-8745-04)

8. A. B. Hummon, S. R. Lim, M. J. Difilippantonio, T. Ried, *Biotechniques* **42** (2007) 467 (https://dx.doi.org/10.2144/000112401)
9. A. E. Simões, D. M. Pereira, J. D. Amaral, A. F. Nunes, S. E. Gomes, P. M. Rodrigues, A. C. Lo, R. D'Hooge, C. J. Steer, S. N .Thibodeau, P. M.Borralho, C. M. P. Rodrigues, *BMC Genomics* **14** (2013) 181 (https://dx.doi.org/10.1186/1471-2164-14-181)
10. A. P. Joy, D. C. Ayre, I. C. Chute, A. P. Beauregard, G. Wajnberg, A. Ghosh, S. M. Lewis, R. J. Ouellette, D. A. Barnett, *J. Extracell. Vesicles* **7** (2018) 1438727 (https://dx.doi.org/10.1080/20013078.2018.1438727)
11. Y. Wen, I. J. Vechetti, T. R. Valentino, J. J. McCarthy, *Biotechniques* **69** (2020) 264 (https://dx.doi.org/10.2144/btn-2020-0083)
12. C. Young, P. Truman, *Anal Biochem.* **421** (2012) 330 (https://doi.org/10.1016/j.ab.2011.10.045)
13. C. Pop, S. Ameling, D. Vishnu, F. Loghin, U. Völker, E. Hammer, *JIOMICS* **5** (2015) 58 (https://dx.doi.org/10.5584/jiomics.v5i1.185)
14. L. Hua, R. Zhou, D. Thirumalai, B.J. Berne, *Proc. Natl. Acad. Sci. USA* **105** (2008) 16928 (https://doi.org/10.1073/pnas.0808427105)
15. L. Kollipara, R.P. Zahedi, *Proteomics* **13** (2013) 941 (https://dx.doi.org/10.1002/pmic.201200452)
16. B. Schlager, A. Straessle, E. Hafen, *BMC Biotechnol.* **12** (2012) (https://dx.doi.org/10.1186/1472-6750-12-95).