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# ISOLATION OF HIGHLY ENRICHED NUCLEAR PROTEOME USING OPTIMIZED METHODS FROM NEUROBLASTOMA CELLS

## *OPTİMİZE EDİLMİŞ YÖNTEMLER KULLANILARAK NÖROBLASTOMA HÜCRELERİNDEN YÜKSEK DERECEDE ZENGİNLEŞTİRİLMİŞ NÜKLEER PROTEOM İZOLASYONU*

Ayimgül Uzunyol[1*], Murat Kasap[2], Gürler Akpınar[2]

[1]OSTIM Technical University, Biomedical Device Technology Faculty, Vocational school, Ankara, Turkiye. [2]Kocaeli University, Department of Medical Biology/DEKART Proteomics Laboratory, Kocaeli University Medical School, Kocaeli, Turkiye.

**ABSTRACT**

**Objective:** Nuclei sits at the center of cells and orchestrates many cellular metabolisim. However, in proteomic studies, we lack of profound understanding of many processes regarding to nuclei due to the poor enrichment of its proteome. In this study, in order to put forward one step in this area of research, comprehensive evaluation of four different nuclear protein enrichment methods were conducted by using neuroblastoma cell lines (SH-SY5Y) as a model.

**Methods:** Nuclear proteins (NPs) have been isolated using either commercially available kits or density gradient centrifugation. The purity of the isolated nuclear proteins have been verified using Western Blot (WB) analysis with antibodies against histonH3, LaminA/C, GAPDH and Cyclophilin A. Further analysis have been performed by using 2-DE (2 dimentioanl gel electrophoresis) gels and the proteins have been identified by MALDI-TOF/TOF analysis.

**Results:** Results from this comparison study demonstrated that Q-Proteome nuclear protein isolation kit (Qiagen, USA) was superior when compared to other most commonly used differential and density gradient centrifugation nuclear protein enrichment methods. Collected fractions using this method gave bands only with anti-histone H3 and anti- LaminA/C antibodies . Approximately 70% of the proteins on 2-DE gels were resident nuclear proteins or predicted to be nuclear-associated.

**Conclusion:** Overall, we demonstrated that although it is not possible to obtain purified nuclear protein fractions, it is feasible to obtain highly enriched nuclear fractions from cells grown in culture. Our study will serve as an important platform for nuclear protein isolation in Protemics.

**Keywords:** Nucleus, Proteomics, neuroblastoma cells, enrichment methods

**ÖZ**

**Amaç:** Çekirdek, hücrelerin merkezinde yer alır ve birçok hücresel metabolizmayı yönetir. Ancak proteomik çalışmalarda, proteomunun zayıf zenginleşmesi nedeniyle çekirdeklerle ilgili birçok işlemi derinlemesine anlamamız mümkün olmamaktadır. Bu çalışmada bu önemli organeli detaylı olarak anlamak için nöroblastoma hücre (SH-SY5Y) model kullanarak dört farklı nükleer protein zenginleştirme yönteminin kapsamlı değerlendirmesi yapılmıştır.

**Yöntem:** Nükleer proteinler (NP'ler), ticari kitler veya yoğunluk gradyanlı santrifüjleme metodu kullanılarak izole edilmiştir. İzole edilmiş nükleer proteinlerin saflığı, histonH3, LaminA/C, GAPDH ve Siklofilin A'ya karşı antikorlarla Western Blot (WB) analizi kullanılarak doğrulanmıştır. 2-DE (2 boyutlu jel elektroforez) jelleri ve proteinler MALDI-TOF/TOF analizi ile tanımlanmıştır.

**Bulgular:** Bu karşılaştırmalı çalışmanın sonuçları, Q-Proteome nükleer protein izolasyon kiti'nin (Qiagen, ABD) diğer en yaygın kullanılan farklı ve yoğunluk gradyanı santrifüjasyonu nükleer protein zenginleştirme yöntemleri ile karşılaştırıldığında üstün olduğunu göstermiştir. Bu yöntemle toplanan fraksiyonlar, yalnızca histon H3 ve Lamin A/C antikorları ile bantlar vermiştir. Yaklaşık olarak 2-DE jellerindeki proteinlerin %70'i yerel nükleer proteinler veya nükleer ile ilişkilendirilmiş olarak tahmin edilmiştir.

**Sonuç:** Genel olarak, saflaştırılmış nükleer protein fraksiyonları elde etmek mümkün olmasa da, kültürde yetiştirilen hücrelerden yüksek derecede zenginleştirilmiş nükleer fraksiyonlar elde etmek mümkündür. Çalışmamız, Proteomik'te nükleer protein izolasyonu için önemli bir platform olarak hizmet edecektir.

**Anahtar Kelimeler:** Çekirdek, Proteomik, nöroblastoma hücreleri, zenginleştirme yöntemleri

***Corresponding author/İletişim kurulacak yazar:** Ayimgül Uzunyol; OSTIM Technical University, Biomedical Device Technology Faculty, Vocational school, Ankara, Turkiye.
**Phone/Telefon:** +90 (531) 263 48 14 **e-mail/e-posta:** ayimgul.uzunyol@ostimteknik.edu.tr
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## Introduction

An organelle is characterized by a subunit of the eukaryotic cells that is specialized in mutiple cellular functions. The subcellular localization of a organelle protein is an paramount factor for protein function. In the same spring, mis-localization of proteins is blame for dysfunction in cell and various human diseases.[1-3] Majority of the organelle proteins are found in the nucleus. Nuclear proteome is characterized by a set of highly dynamic and multiple overlapping structure. Apporximately 1425 protein-coding genes have been shown to located in nucleoli. In recent years, high-throughput methods have been developed based on either enrichment or isolation for organelle proteome and the most commonly used approach is subcellular fractionation.[4-6] The first procedure for subcellular fractionation is disruption of cells and tissues using physical methods such as mechanical or liquid shear or non-physical methods such as detergents or hypo-osmotic shock. The cell disruption step is aimed at obtaining high protein yields, and maintain structural and functional integrity of intracellular organelles. The conventional methods such as subcellular fractionation which are typically by differential and density-gradient centrifugation, have been employed a series of centrifugation steps to separate different populations of cellular compartments or organelles from cell homogenates based on their mass and/or density.[7] Eventhough more than 150 years of research into nucleoli, many aspects of their structure and function remain uncharacterized as a black whole. The noteyworthy tendency in nucleoli research currently is rapid advances in nuclear proteomes research which allow us to deeply understand the nucleus-related metabolism in plants and animals cells. The most popular method for isolation of nuclei from cells was developed by Blobel and Potter in 1966.[8] The basic principle of the protocol is relied on high sedimentation velocity of nuclei relative to all other components in the somatic cell. The purification of nuclei from mammalian cells is associated with many technical problems such as time control of cell swelling using as hypotonic buffer, temperature control during mechanical disruption methods and washing times of the cell lysis. While excessive hypotonic swelling leads to nuclear membrane disruption, hypertonic buffers cause nuclear shrinking and therefore alter the density and lumen volume of the isolated nuclei potentially affecting their integrity and protein content. Temperature control is hardly be assured at any time of the nuclear isolation procedure during the mechanical disruption methods and as a result of uncontrolled temperature the nuclei are in extensive contact with the cytoplasmic environment containing lysis-activated proteases which lead to partial degradation and modification of the samples despite the presence of protease inhibitors.[9,10] Repeated washing steps are necessary due to leakage of the nucleus-associated membrane systems and cytoskeletal components by overexposure to detergent and mechanical stress. Since nuclear leakage may lead to not only in loss of target proteins but also in clumping of the nuclei due to released chromosomal DNA.[11]

In this study, NPs from SH-SY5Y cells have been enriched by using four different methods namely, (1) Extraction of NPs using ReadyPrep™ Protein Extraction Kit, (2) Isolation of NPs using OptiPrep Application sheet, (3) An improved method using discontinues density gradient centrifugation, (4) Qproteome Nuclear Protein enrichment kit. The efficiencies of the methods have been evaluated by using the antibodies against nuclear proteins (NPs), histonH3, LaminA/C, beta-actin (β-actin) or glyceraldehyde-3-phosphate dehydrogenase (GAPDH). Quantitative evaluation of the level of enrichment has been assessed by 2-DE gels coupled to MALDI-TOF/TOF analysis. In our study, the comparison of enrichment metods have demonstrated that enrichment of NPs using Qproteome Nuclear Protein enrichment kit approach was more successful than the other methods for the identifcation of NPs.

## Methods

### Cell culture

SH-SY5Y cells were cultured under standard tissue culture conditions as follows: cells were grown in EMEM supplemented with 10% (Vol/Vol) tetracycline-reduced fetal bovine serum, 100 U/ml penicillin streptomycin and 2mML-glutamine at 37 °C in a humidified 5% CO2 atmosphere, cells were cultured until reach to 90 % of confluence.

### Enrichment of Nuclear Protein Extracts

#### *Enrichment of NPs From Cultured Cells using ReadyPrep (Cytoplasmic/Nuclear) Protein Extraction Kit*

The ReadyPrep Protein Extraction Kit (Cytoplasmic/Nuclear) provides a buffer/differential centrifugation protocol to generate enriched cytoplasmic fractions and intact nuclei.[12,13] The procedure of enrichment was followed instructions provided by the manufacturer (Bio-Rad, USA, ReadyPrep™ Protein Extraction Kit, #163-2089). In brief, 0.5 ml of packed cells were resuspended 0.5 ml cytoplasmic protein extraction

buffer (CPEB) for 30 min and then were lysed by gently passing of the cell suspension through a narrow opening of a 20 gauge needle without damaging the nuclei. The cell lysate was centrifuged at 1000 xg for 10 min at 4°C and the cytoplasmic proteins in the supernatant were collected. The pellet was washed several times with CPEB and nuclear proteins were then solubilized with a strongly chaotropic extraction buffer.

***Enrichment of nuclei from cultured cells using OptiPrep medium***

The commercially available form of discontinuous iodixanol gradient medium, OptiPrep-(SigmaAldrich, USA) allows enrichment of nuclei from cultured cells by isopycnic banding in an iso-osmotic environment.[14] This method is based on the principle that sedimentation of the particles is very slow due to the high viscosity of the iodixanol (=1.32 g/ml) barrier. The cell homogenate (Adjusted to approx =1.14 g/ml) in 25% iodixanol was prepared according to the sheet instructions provided by the manufacturer, then the homogenate sample was underlayered with 15 ml of the 30% iodixanol (Adjusted to approx =1.175 g/ml) and 15 ml of the 35% iodixanol(Adjusted to approx = 1.20 g/ml ). In the last step, the gradient system consist of three bands was placed in a swinging-bucket rotor of a high-speed centrifuge for centrifigation at 10,000xg for 20 min and the band of nuclei at the 30%-35% iodixanol interface was colleted (Figure1). The fractions were clean-up using a commercial kit by Biorad (The ReadyPrep 2-D cleanup kit, # 1632130).

***Enrichment of NPs from cultured cells using Q-proteome nuclear protein enrichment approach***

A commercial kit by Qiagen (Q-proteome Nuclear Protein enrichment kit, # 37531) was used to enrich NPs. All steps were performed according to the instructions provided by the manufacturer. The approach relies on incubation of the cells in hypotonic buffer, causing them to swell. Then, detergent added to the lysis buffer to rupture the plasma membrane and centrifugation is used to separate the cytosolic fraction from the cell nuclei. In brief, starting material 5 x $10^6$ –1 x $10^7$ SH-SY5Y cells were grown in T75 flasks to 80% confluency and the harvested cell pellet was resuspended in 500 µl lysis buffer NL containing Protease Inhibitor Solution and 0.1 M DTT by pipetting up and down and incubated on ice for 15 min. 25 µl of detergent solution NP was added and centrifuged to collect the nuclear pellet. The nuclear pellet was then resuspend with 50 µl extraction buffer and disrupted by gentle agitation in a thermomixer (SigmaAldrich, USA) at 4°C and centrifuged to collect supernatant of nucleic-acid binding proteins (NABP). 150 µl of dilution buffer ND containing Protease Inhibitor Solution and 0.1 M DTT was added to the NABP and further purification was proceeded using Nuclear Protein Fractionation Column 500 µl of elution buffer NE1, elution buffer NE2 and elution buffer NE3 respectively. The “insoluble” nuclear proteins (NIPs) were achieved by incubation and centrifugation of resuspended pellet in 100 µl Extraction Buffer NX2.

***Enrichment of NPs from cultured cells using discontinues density gradient centrifugation (DDGC)***

Before DDGC, crude nuclei were isolated using The ReadyPrep Protein Extraction Kit (Cytoplasmic/Nuclear). The cleaner nuclei were isolated following the protocol described in Graham et al[14] . In brief, SH-SY5Y cells were resuspended in 2 mL of buffer A (10 mM HEPES, pH 7.4, 10 mM NaCl, 3 mM MgCl2, 1 mM EGTA, 0.05% Triton X-100) and homogenized by pipetting. The cell homogenate was pelleted through adding 4 mL of 250 mM sucrose in buffer B (50 mM HEPES, pH 7.4, 50 mM NaCl, 5 mM MgCl2, 1 mM EGTA) to cell suspension at 1500xg for 10 min to obtain crude nuclei. The crude nuclear pellet was the resuspended in 3 mL of 25% iodixanol and 250 mM sucrose in buffer B and placed on top of a iodixanol step gradient consisting of 4.5 mL of 25% iodixanol, 15 mL of 30% iodixanol, and 15 mL of 35% iodixanol in buffer B (without sucrose) for ultracentrifugation at 10 000xg for 20 min. The isopycnic band at the 30−35% iodixanol interface contained the clean nuclei. Solublized with 2D rehydration buffer.

***Determination of protein concentration***

Bradford Assay was used to measure total protein concentration in cell-free extracts.[15] In brief, 1 µL of protein extract was mixed with 19 µL sample preparation buffer. The mixture was vortexed with 1mL of 1x Bradford Reagent and allowed to sit for 5 min in the dark. The samples were then measured at 595 nm with Nanodrop. The measured protein concentrations of the samples were determined by comparing with the standards curve prepared using BSA. The BSA standards were prepared in the same buffer as the unknown protein samples.

***Western blot analysis***

NPs were verified using Western Blot (WB) analysis with antibodies against histonH3(histonH3, Cell Signaling Technology, USA), LaminA/C (Lamin A/C (JoL3,): sc-56140, Santa Cruz, USA), Glyceraldehyde-3-phosphate dehydrogenase (GAPDH (6C5): sc-32233, Santa Cruz,

USA) and Cyclophilin A (PPIA - peptidylprolyl isomerase A, Novus Biologicals, USA).

NPs were fractionated on 12% SDS-PAGE and electrophoretically transferred onto nitrocellulose sheets. Membranes were blocked in 5% nonfat dry milk in membranes were treated with 5% non-fat dry milk/Tris buffered saline (TBS, 100 mM Tris-HCl, 150 mM NaCl, pH 7.6) containing 0.1% vol/vol Tween20 for 1 h and then incubated with indicated antibodies overnight at 4°C. Membranes were washed three times with TBST with 0.1% v/v Tween 20, and then incubated with HRP-conjugated secondary antibody. After washing, immune complexes were detected by reaction in the ECL assay (BioRad, USA) according to the manufacturer.

***Two-Dimensional gels electrophoresis (2DE)***

The protein concentrations of nuclear extracts were measured by Bradford Protein Assay (BioRad, USA). About 200 µg of protein was loaded onto each IPG strip (11cm, pH 5-8, BioRad, USA) and actively (50V) rehydrated for 16 hrs. The protein-loaded strips were focused on Protean IEF cell by using the recommended focusing conditions (BioRad, USA). Following focusing, strips were washed with equilibration buffer I (6M urea, 2% SDS, 37 mM Tris-HCl, pH 8.8, 20% glycerol) containing 20 mg/mL of DTT for 15 min at 22°C with shaking. Afterward the samples were then incubated in another 4 mL of equilibration buffer II (6M urea, 2% SDS, 37 mM Tris-HCl, pH 8.8, 20% glycerol) with 25 mg/mL of iodoacetamide/mL for 15 min at 227C with shaking. After a final wash in SDS-PAGE running buffer, strips were used in SDS-PAGE for separation in the second dimension. (BioRad, USA) to prevent gel to gel variations. The runs were ended when the front dye reached to the bottom of the gels. The gels were fixed in 40% methanol plus 10% acetic acid solution for at least 8 hr and then were stained overnight in Coomassie Brilliant Blue G-250 (BioRad, USA). VersaDoc4000MP was used for imaging and the images were analyzed with PDQuest Advance (BioRad, USA). Spots were cut by using an ExQuest spot cutter and identified by MALDI-TOF/TOF (Applied Biomics, USA).

***Image Analysis***

2D gel images were captured with VersaDoc 4000MP system by using Quantity One softwaree (Bio-Rad, USA- Version 4.6.7). 2D gel image comparison, protein spot intensities with more than two-fold significant-change ($p<0.05$) in a consistently increased or decreased pattern were considered differentially expressed. All selected spots were excised using an automated spot cutting tool, ExQuest Spot Cutter using PDQuest Advanced 2-D analysis software (Bio-Rad, USA- Version 8.0.1), and disposed into 96-well plates for identification.

***Protein Identifcation***

All experiments related to Protein identification have been performed at Kocaeli University Medical Biology Proteomics Laboratory (Kocaeli, Turkey) using ABSCIEX MALDI-TOF/TOF 5800 system (Applied Biosystems®, Framingham, MA, USA). Protein spots cutted by the spot-cutter device in 96-well plates were replaced into 0.6 mL microcentifuge tubes and a commercially aviable kit (Pierce®, USA). was used for in-gel tryptic digestion. All steps of digestion was followed protocol provided by manufacturer prior to the desaltaion of samples with a 10 µl ZipTipC18 (Millipore®, USA). The peptides were eluted with α-cyano-4-hydroxycinnamic acid solution (α-CHCA) in 0.1% trifuoroacetic acid(TFA)/50% acetonitrile as the matrix (1:1, v/v) and spotted (~0.5 µL) directly onto a stainless-steel MALDI target plate. Protein identification experiments were performed by using ABSCIEX MALDI-TOF/TOF 5800 system. The TOF spectra were recorded in the positive ion reflector mode with a mass range from 400 to 2000 Da. Each spectrum was the cumulative average of 2000 laser shots. The spectra were calibrated with the trypsin autodigestion ion peaks m/z (842.510 and 2211.1046) as internal standards. Ten of the strongest peaks of the TOF spectra per sample were chosen for MS/MS analysis. All of the PMFs were searched in the MASCOT version 2.5(Matrix Science) by using a streamline software, Protein Pilot (ABSCIEX®, USA), with the following criteria: SWISSPROT database; species restriction to H. sapiens; enzyme of trypsin; at least ten independent peptides matched; at most one missed cleavage site; MS tolerance set to ±50 ppm and MS/MS tolerance set to ±0.2 Da; fixed modification being carbamidomethyl (Cys) and variable modification being oxidation (Met); peptide charge of +1 and being monoisotopic. Only significant hits, as defined by the MASCOT probability analysis ($p<0.05$), were accepted.

## Results

Four diferent commonly used NPs enrichment methods have been evaluated to provide a comparative assessment for isolation of NPs. The isolated proteins have been then seperated and identified by 2DE coupled to MALDI-TOF/TOF (Tables 1 and 2).

**Table 1.** List of identified proteins using different methods by MALDI-TOF/TOF

| No. | AC no. | Protein Name | Protein Score | Expect | Matches | pI | Seq. Cov. (%) | Subcellular Location | Methods of enrichment |
|---|---|---|---|---|---|---|---|---|---|
| Nuclear proteins | | | | | | | | | |
| 1 | O96019 | Actin-like protein 6A | 122 | 1.30e-08 | 9 | 5.39 | 16 | Nucleus | Improved |
| 2 | P20700 | Lamin-B1 | 539 | 2,6E-50 | 38 | 5.11 | 40 | Nucleus inner membrane | Improved |
| 3 | Q12912 | Inositol 1,4,5-triphosphate receptor associated 2 | 56 | 0,049 | 7 | 5.62 | 8 | Nucleus envelope | Improved |
| 4 | Q03252 | Lamin-B2 | 579 | 2,6E-54 | 42 | 5.29 | 45 | Nucleus inner membrane | Improved |
| 5 | Q09028 | Histone-binding protein RBBP4 | 70 | 0,0022 | 2 | 4,74 | 3 | Nucleus | Improved |
| 6 | Q9Y230 | RuvB-like 2 | 102 | 1.30e-06 | 9 | 5,49 | 22 | Nucleus matrix | Improved |
| 7 | P38919 | Eukaryotic initiation factor 4A-III | 628 | 3,2E-59 | 30 | 6,3 | 35 | Nucleus | Improved |
| 8 | P36873 | Serine/threonine-protein phosphatase PP1-gamma catalytic subunit | 55 | 0,063 | 6 | 6,13 | 13 | Nucleus | Improved |
| 9 | Q15366 | Poly(rC)-binding protein 2 | 74 | 7, 7E-04 | 5 | 6,33 | 13 | Nucleus | Improved |
| 10 | P53582 | Methionine aminopeptidase 1 | 96 | 4,80E-06 | 8 | 6,75 | 24 | Nucleus | Improved |
| 11 | P18754 | Regulator of chromosome condensation | 228 | 3,20E-19 | 12 | 7,18 | 18 | Nucleus | Improved |
| 12 | Q9NVX2 | Notchless protein homolog 1 | 60 | 0,023 | 9 | 6,92 | 13 | Nucleus | Improved |
| 13 | Q2TAY7 | WD40 repeat-containing protein SMU1 SMU1_HUMAN | 566 | 5,1E-53 | 21 | 6,74 | 25 | Nucleus | Improved |
| 14 | P14866 | Heterogeneous nuclear ribonucleoprotein L | 279 | 2,6E-24 | 25 | 8,46 | 21 | Nucleus | Improved |
| 15 | P12956 | X-ray repair cross-complementing protein 6 | 127 | 4,0E-09 | 10 | 6,23 | 8 | Nucleus | Improved |
| 16 | O43143 | ATP-dependent RNA helicase DHX15 | 66 | 0,0057 | 5 | 7,12 | 4 | Nucleus | Improved |
| 17 | P07910 | Heterogeneous nuclear ribonucleoproteins C1/C2 | 157 | 4, 0E-12 | 15 | 4,95 | 20 | Nucleus | Improved |
| 18 | P63244 | Guanine nucleotide-binding protein subunit beta-2-like 1 | 312 | 1, 30E-27 | 13 | 7,6 | 21 | Nucleus | Improved |
| 19 | P26583 | High mobility group protein B2 | 63 | 0,0099 | 7 | 7,62 | 22 | Nucleus | Improved |
| 20 | Q14103 | Heterogeneous nuclear ribonucleoprotein D0 | 107 | 4,0E-07 | 4 | 7,62 | 12 | Nucleus | Improved |
| 21 | Q07666 | KH domain-containing, RNA-binding, signal transduction-associated protein 1 | 56 | 0,055 | 9 | 8,73 | 13 | Nucleus | Improved |
| 22 | P51991 | Heterogeneous nuclear ribonucleoprotein A3 | 97 | 4,0E-06 | 8 | 9,1 | 14 | Nucleus | Improved |
| 23 | P09651 | Heterogeneous nuclear ribonucleoprotein A1 | 266 | 5,10E-23 | 266 | 9,17 | 31 | Nucleus | Improved |
| 24 | O15226 | NF-kappa-B-repressing factor | 68 | 0,0034 | 24 | 8,94 | 22 | Nucleus | Improved |
| 25 | P31943 | Heterogeneous nuclear ribonucleoprotein H | 150 | 2,00E-11 | 16 | 5,89 | 24 | Nucleus | Improved |
| 26 | P17844 | Probable ATP-dependent RNA helicase DDX5 | 58 | 0,029 | 19 | 9,06 | 23 | Nucleus | Improved |
| 27 | P38159 | Heterogeneous nuclear ribonucleoprotein G | 139 | 2,60E-10 | 24 | 10,06 | 43 | Nucleus | Improved |

| | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| 28 | P07910 | Heterogeneous nuclear ribonucleoproteins C1/C2 | 88 | 3,40E-05 | 12 | 4,95 | 25 | Nucleus | Improved |
| 29 | P22626 | Heterogeneous nuclear ribonucleoproteins A2/B1 | 332 | 1,30E-29 | 22 | 8,97 | 42 | Nucleus | Improved |
| 30 | Q92804 | TATA-binding protein-associated factor 2N | 87 | 4, 0E -05 | 16 | 8,04 | 30 | Nucleus | QProteome -NABPF |
| 31 | Q96AE4 | Far upstream element-binding protein 1 | 453 | 1, 0E-41 | 34 | 7,18 | 38 | Nucleus | QProteome -NABPF |
| 32 | O43172 | U4/U6 small nuclear ribonucleoprotein Prp4 | 194 | 8.1E-16 | 26 | 7,71 | 38 | Nucleus | QProteome -NABPF |
| 33 | P14866 | Heterogeneous nuclear ribonucleoprotein L | 289 | 2.60E-25 | 32 | 8,46 | 42 | Nucleus | QProteome -NABPF, Opti prep |
| 34 | Q92945 | Far upstream element-binding protein 2 | 353 | 1.00E-31 | 36 | 6,84 | 41 | Nucleus | QProteome -NABPF |
| 35 | Q09028 | Histone-binding protein RBBP4 | 134 | 8,10E-10 | 17 | 4,74 | 23 | Nucleus | QProteome -NIPF |
| 36 | P08865 | 40S ribosomal protein SA | 117 | 4,00E-08 | 6 | 4,79 | 16 | Nucleus | QProteome -NIPF |
| 37 | Q13610 | Periodic tryptophan protein 1 homolog | 114 | 8,10E-08 | 15 | 4,6 | 13 | Nucleus | QProteome-NIPF |
| 38 | P43243 | MATR3 | 57 | 0,042 | 15 | 5,87 | 13 | Nucleus matrix | Opti prep |
| 39 | P23246 | Splicing factor, proline- and glutamine-rich | 380 | 2,00E-34 | 38 | 9,45 | 41 | Nucleus | Opti prep |
| Cytoplasmic proteins | | | | | | | | | |
| 40 | P08670 | Vimentin | 618 | 3,2E-58 | 37 | 5.06 | 48 | Cytoplasm | Biorad, Improved, QProteome -NIPF |
| 41 | P04264 | Keratin, type II cytoskeletal 1 | 484 | 8,1E-45 | 12 | 8,15 | 27 | Cytoplasm | Improved, Opti prep |
| 42 | P11142 | Heat shock cognate 71 kDa protein | 540 | 2.00E-50 | 24 | 5,37 | 30 | Cytoplasm | Biorad, Improved |
| 43 | P34931 | Heat shock 70 kDa protein 1-like | 101 | 1,60E-06 | 20 | 5,76 | 25 | Cytoplasm | Improved |
| 44 | P07437 | Tubulin beta chain | 170 | 2,00E-13 | 22 | 4,78 | 26 | Cytoplasm | QProteome -NIPF |
| 45 | P68363 | Tubulin alpha-1B chain | 179 | 2,60E-14 | 25 | 4,94 | 45 | Cytoplasm | QProteome -NIPF |
| 44 | Q7Z7B0 | Filamin-A-interacting protein 1 | 58 | 0,035 | 28 | 8,46 | 23 | Cytoplasm | QProteome -NIPF |
| 46 | P60174 | Triosephosphate isomerase | 517 | 4,00E-48 | 21 | 6,45 | 54 | Cytoplasm | Opti prep |
| Mitochondrian proteins | | | | | | | | | |
| 47 | P10809 | 60 kDa heat shock protein | 130 | 2,0E-09 | 13 | 5.7 | 21 | Mitichondrian matrix | Biorad |
| 48 | P06576 | ATP synthase subunit beta | 262 | 1,3E-22 | 16 | 5,26 | 29 | Mitichondrian matrix | Biorad , Improved |
| 49 | P25705 | ATP synthase subunit alpha | 187 | 4,0E-15 | 8 | 9,16 | 8 | Mitochondrial membrane | Improved |
| 50 | P35232 | Prohibitin | 86 | 5,0E-05 | 9 | 5,57 | 26 | Mitichondrian inner membrane | Improved |
| 51 | P34897 | Serine hydroxymethyltransferase | 169 | 2,60E-13 | 22 | 8,76 | 33 | Mitichondrian matrix | Opti prep |
| 52 | P09622 | Dihydrolipoyl dehydrogenase | 243 | 1,00E-20 | 22 | 7,95 | 30 | Mitichondrian matrix | Opti prep |
| 53 | Q07021 | Complement component 1 Q subcomponent-binding protein | 89 | 2,60E-05 | 5 | 4,74 | 21 | Mitichondrial | Opti prep |
| Endoplasmic reticulum proteins | | | | | | | | | |
| 54 | P11021 | Endoplasmic reticulum chaperone BiP | 232 | 1,3E-19 | 16 | 5,07 | 18 | ER lumen | Improved |

| | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| 55 | P07237 | Protein disulfide-isomerase | 190 | 2.00e-15 | 9 | 4,76 | 13 | Endoplasmic reticulum | Improved |
| 56 | P27797 | Calreticulin | 102 | 1.30e-06 | 7 | 4,29 | 14 | Endoplasmic reticulum | Improved |
| 57 | P30101 | Protein disulfide-isomerase A3 | 182 | 1.30e-14 | 25 | 5,98 | 28 | Endoplasmic reticulum | Improved |
| Other proteins | | | | | | | | | |
| 58 | P08107 | Heat shock 70 kDa protein 1A/1B | 121 | 1.60e-08 | 17 | 5.48 | 23 | Unknown | Biorad |
| 59 | P34931 | Heat shock 70 kDa protein 1-like | 266 | 5,1E-23 | 18 | 5,76 | 23 | Blood microparticle, cytosol | Improved |

**Table 2.** List of identified proteins using QProteome method by MALDI-TOF/TOF

| No. | AC no. | Protein Name | Protein Score | Expect | Matches | pI | Seq. Cov. (%) | Subcellular Location | Methods of enrichment |
|---|---|---|---|---|---|---|---|---|---|
| Nuclear proteins | | | | | | | | | |
| 1 | P38159 | Mediator of RNA polymerase II transcription subunit 11 | 139 | 2.60e-10 | 24 | 10.06 | 43 | Nucleus | QProteome |
| 2 | P07910 | Heterogeneous nuclear ribonucleoproteins C1/C2 | 234 | 8.10e-20 | 17 | 4.95 | 25 | Nucleus | QProteome |
| 3 | P22626 | Heterogeneous nuclear ribonucleoproteins A2/B1 | 332 | 1.30e-29 | 22 | 8.97 | 42 | Nucleus | QProteome |
| 4 | P35232 | Prohibitin | 95 | 6.10e-06 | 12 | 5.57 | 37 | Nucleus | QProteome |
| 5 | Q92804 | TATA-binding protein-associated factor 2N | 87 | 4.00e-05 | 16 | 8.04 | 30 | Nucleus | QProteome |
| 6 | Q96AE4 | Far upstream element-binding protein 1 | 453 | 1.00e-41 | 34 | 7.18 | 34 | Nucleus | QProteome |
| 7 | O43172 | U4/U6 small nuclear ribonucleoprotein Prp4 | 194 | 8.10e-16 | 26 | 7.705 | 38 | Nucleus | QProteome |
| 8 | P14866 | Heterogeneous nuclear ribonucleoprotein L | 209 | 2.60e-17 | 30 | 8.46 | 37 | Nucleus | QProteome |
| 9 | Q92945 | Far upstream element-binding protein 2 | 353 | 1.00e-31 | 36 | 6.84 | 41 | Nucleus | QProteome |
| 10 | Q09028 | Histone-binding protein RBBP4 | 134 | 8.10e-10 | 17 | 4.74 | 23 | Nucleus | QProteome |
| 11 | Q16576 | Histone-binding protein RBBP7 | 95 | 6.10e-06 | 22 | 4.89 | 29 | Nucleus | QProteome |
| 12 | P08865 | 40S ribosomal protein SA | 117 | 4.00e-08 | 17 | 4.74 | 23 | Nucleus | QProteome |
| 13 | Q13610 | Periodic tryptophan protein 1 | 114 | 8.10e-08 | 15 | 4.60 | 13 | Nucleus | QProteome |
| 14 | P13639 | Elongation factor 2 | 154 | 8.10e-12 | 30 | 6.41 | 30 | Nucleus | QProteome |
| 15 | Q9Y265 | RuvB-like 1 | 166 | 5.10e-13 | 30 | 6.02 | 48 | Nucleus | QProteome |
| 16 | P31943 | Heterogeneous nuclear ribonucleoprotein H | 186 | 5.10e-15 | 24 | 5.89 | 53 | Nucleus | QProteome |
| 17 | P06748 | Nucleophosmin | 160 | 2.00e-12 | 14 | 4.64 | 30 | Nucleus | QProteome |
| 18 | P11142 | Heat shock cognate 71 kDa protein | 153 | 1.00e-11 | 23 | 5.37 | 35 | Nucleus | QProteome |
| 19 | Q6ZNG1 | Zinc finger protein 600 | 66 | 0,0048 | 25 | 9.41 | 28 | Nucleus | QProteom |

| | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| 20 | P26641 | Elongation factor 1-gamma | 118 | 3.20e-08 | 18 | 6.25 | 32 | Nucleus | QProteome |
| 21 | P29692 | Elongation factor 1-delta | 135 | 6.40e-10 | 18 | 4.90 | 38 | Nucleus | QProteome |
| 22 | P45973 | Chromobox protein homolog 5 | 95 | 6.10e-06 | 16 | 5.71 | 69 | Nucleus | QProteome |
| 23 | Q9Y230 | RuvB-like 2 | 207 | 4.00e-17 | 39 | 5.49 | 50 | Nucleus, cytoplasm | QProteome |
| 24 | Q96J01 | THO complex subunit 3 | 69 | 0.0027 | 10 | 5.7 | 21 | Nucleus | QProteome |
| 25 | P09429 | High mobility group protein B1 | 73 | 0.00095 | 14 | 5.62 | 42 | Nucleus | QProteome |
| 26 | Q15365 | Poly(rC)-binding protein 1 | 97 | 3.80e-06 | 13 | 6.66 | 48 | Nucleus | QProteome |
| 27 | P60174 | Triosephosphate isomerase | 245 | 6.40e-21 | 24 | 6.45 | 75 | Nucleus | QProteome |
| 28 | P45880 | Voltage-dependent anion-selective channel protein 2 | 113 | 1.00e-07 | 11 | 7.49 | 43 | Nucleus | QProteome |
| 29 | P07355 | Annexin A2 | 148 | 3.20e-11 | 24 | 7.57 | 53 | Nucleus | QProteome |
| 30 | P12004 | Proliferating cell nuclear antigen | 105 | 6.40e-07 | 12 | 4.57 | 43 | Nucleus | QProteome |
| 31 | P19338 | Nucleolin | 109 | 2.60e-07 | 23 | 4.6 | 27 | Nucleus | QProteome |
| 32 | Q12874 | Splicing factor 3A subunit 3 | 134 | 8.10e-10 | 22 | 5.27 | 36 | Nucleus | QProteome |
| 33 | P08865 | 40S ribosomal protein SA | 67 | 0.0043 | 16 | 4.79 | 35 | Nucleus | QProteome |
| 34 | P31942 | Heterogeneous nuclear ribonucleoprotein H3 | 330 | 2.00e-29 | 32 | 6.37 | 77 | Nucleus | QProteome |
| 35 | P08238 | Heat shock protein HSP 90-beta | 151 | 1.60e-11 | 33 | 4.97 | 33 | Nucleus | QProteome |
| 36 | Q9P258 | Protein RCC2 | 193 | 1.00e-15 | 25 | 9.02 | 33 | Nucleus | QProteome |
| 37 | P29692 | Elongation factor 1-delta | 135 | 6.40e-10 | 18 | 4.9 | 38 | Nucleus | QProteome |
| 38 | O95831 | Apoptosis-inducing factor 1 | 92 | 1.40e-05 | 22 | 9.04 | 31 | Nucleus | QProteome |
| 39 | Q9P258 | Protein RCC2 | 193 | 1.00e-15 | 25 | 9.02 | 33 | Nucleus | QProteome |
| 40 | Q07955 | Splicing factor, arginine/serine-rich 1 | 225 | 6.40e-19 | 22 | 10.37 | 44 | Nucleus | QProteome |
| 41 | P61978 | Heterogeneous nuclear ribonucleoprotein K | 260 | 2.00e-22 | 24 | 5.39 | 41 | Nucleus | QProteome |
| 42 | Q12874 | Splicing factor 3A subunit 3 | 108 | 3.20e-07 | 20 | 5.27 | 32 | Nucleus | QProteome |
| 43 | P13838 | Spliceosome RNA helicase BAT1 | 306 | 5.10e-27 | 30 | 5.44 | 41 | Nucleus | QProteome |
| 44 | P14866 | Heterogeneous nuclear ribonucleoprotein L | 174 | 8.10e-14 | 26 | 8.46 | 31 | Nucleus | QProteome |
| 45 | P34897 | Serine hydroxymethyltransferase | 64 | 0.0086 | 16 | 8.76 | 22 | Nucleus | QProteome |
| 46 | P12956 | ATP-dependent DNA helicase 2 subunit 1 | 328 | 3.20e-29 | 20 | 6.23 | 23 | Nucleus | QProteome |
| 47 | O00148 | ATP-dependent RNA helicase DDX39 | 160 | 2.00e-12 | 19 | 5.46 | 25 | Nucleus | QProteome |
| Cytoplasmic proteins | | | | | | | | | |
| 48 | P08670 | Vimentin1 | 313 | 1.00e-27 | 49 | 5.06 | 68 | Cytoplasm | QProteome |
| 49 | P60174 | Triosephosphate isomerase | 278 | 3.20e-24 | 27 | 6.45 | 80 | Cytoplasm | QProteome |
| 50 | P15121 | Aldose reductase | 172 | 1.30e-13 | 24 | 6.51 | 51 | Cytoplasm | QProteome |
| 51 | P60709 | Actin | 161 | 1.60e-12 | 18 | 5.29 | 54 | Cytoplasm | QProteome |

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|---|---|---|---|---|---|---|---|---|---|
| 52 | P08238 | Heat shock protein HSP 90-beta | 151 | 1.60e-11 | 33 | 4.97 | 33 | Cytoplasm | QProteome |
| 53 | P26641 | Elongation factor 1-gamma | 118 | 3.20e-08 | 18 | 6.25 | 32 | Cytoplasm | QProteome |
| 54 | P40227 | T-complex protein 1 subunit zeta | 147 | 4.00e-11 | 22 | 6.23 | 30 | Cytoplasm | QProteome |
| 55 | P32119 | Peroxiredoxin-2 | 104 | 8.10e-07 | 10 | 5.66 | 36 | Cytoplasm | QProteome |
| 56 | P60842 | Eukaryotic initiation factor 4A-I | 148 | 3.20e-11 | 26 | 5.32 | 48 | Cytoplasm | QProteome |
| 57 | P17174 | Aspartate aminotransferase | 57 | 0.04 | 10 | 6.52 | 20 | Cytoplasm | QProteome |
| 58 | P63241 | Eukaryotic translation initiation factor 5A-1 | 57 | 0.039 | 7 | 5.08 | 27 | Cytoplasm,Nucleus, Endoplasmic reticulum | QProteome |
| 59 | P13645 | Keratin, type I cytoskeletal 10 | 137 | 4.00e-10 | 25 | 5.13 | 25 | Cytoplasm | QProteome |
| 60 | P35527 | Keratin, type I cytoskeletal 9 | 91 | 1.60e-05 | 23 | 5.14 | 35 | Cytoplasm, | QProteome |
| 61 | P08758 | Annexin A5 | 437 | 4.00e-40 | 23 | 4.94 | 56 | Cytoplasm | QProteome |
| 62 | P04264 | Keratin, type II cytoskeletal 1 | 213 | 1.00e-17 | 26 | 8.15 | 34 | Cytoplasm, | QProteome |
| 63 | P07195 | L-lactate dehydrogenase B chain | 193 | 1.00e-15 | 20 | 5.71 | 48 | Cytoplasm | QProteome |
| 64 | P47756 | F-actin-capping protein subunit beta | 91 | 1.50e-05 | 16 | 5.36 | 29 | Cytoplasm, | QProteome |
| 65 | P30041 | Peroxiredoxin-6 | 261 | 1.60e-22 | 22 | 6.00 | 57 | Cytoplasm, | QProteome |
| 66 | P06733 | Alpha-enolase | 379 | 2.60e-34 | 29 | 7.01 | 45 | Cytoplasm | QProteome |
| 67 | P04075 | Fructose-bisphosphate aldolase A | 163 | 1.00e-12 | 20 | 8.3 | 40 | Cytoplasm | QProteome |
| 68 | Q15019 | Septin-2 | 80 | 0.0002 | 17 | 6.15 | 44 | Cytoplasm | QProteome |
| 69 | P13693 | Translationally-controlled tumor protein | 131 | 1.60e-09 | 16 | 4.84 | 50 | Cytoplasm | QProteome |
| 70 | P47813 | Eukaryotic translation initiation factor 1A | 64 | 0.0079 | 6 | 5.07 | 30 | Cytoplasm | QProteome |
| 71 | Q07955 | Splicing factor, arginine/serine-rich 1 | 225 | 6.40e-19 | 22 | 10.37 | 44 | Cytoplasm,nucleus | QProteome |
| 72 | P05198 | Eukaryotic translation initiation factor 2 subunit 1 | 149 | 2.60e-11 | 26 | 5.02 | 50 | Cytoplasm | QProteome |
| 73 | Q01105 | Protein SET | 57 | 0.042 | 12 | 4.23 | 31 | Cytoplasm, ER,nucleus | QProteome |
| 74 | P12277 | Creatine kinase B-type | 278 | 3.20e-24 | 36 | 5.34 | 59 | Cytoplasm, ER | QProteome |
| 75 | Q13347 | Ukaryotic translation initiation factor 3 subunit I | 74 | 0.00083 | 17 | 6.4 | 22 | Cytoplasm | QProteome |
| 76 | P61158 | Actin-related protein 3 | 63 | 0.011 | 12 | 5.61 | 24 | Cytoplasm, nucleus | QProteome |
| ER proteins | | | | | | | | | |
| 77 | P07237 | Protein disulfide-isomerase | 366 | 5.10e-33 | 28 | 4.76 | 39 | Endoplasmic reticulum | QProteome |
| 78 | Q13162 | Peroxiredoxin-4 | 283 | 1.00e-24 | 18 | 5.86 | 43 | Endoplasmic reticulum | QProteome |
| 79 | P11021 | 78 kDa glucose-regulated protein | 573 | 4.00e-50 | 48 | 5.07 | 54 | Endoplasmic reticulum lumen | QProteome |
| 51 | P14625 | Endoplasmin | 408 | 3.20e-37 | 42 | 4.76 | 32 | Endoplasmic reticulum | QProteome |
| 80 | P30101 | Protein disulfide-isomerase A3 | 255 | 6.40e-22 | 31 | 5.98 | 42 | Endoplasmic reticulum,lumen,melonesome | QProteome |
| 81 | P27797 | Calreticulin | 123 | 1.00e-08 | 21 | 4.29 | 45 | Cytoplasm | QProteome |

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|---|---|---|---|---|---|---|---|---|---|
| 82 | P67809 | Nuclease-sensitive element-binding protein 1 | 56 | 5.30e-02 | 14 | 9.87 | 23 | Endoplasmic reticulum | QProteome |
| 83 | Q15084 | Protein disulfide-isomerase A6 | 237 | 4.00e-20 | 20 | 4.95 | 33 | Endoplasmic reticulum,lumen,cell membrane,melonesome | QProteome |
| 84 | 9BS26 | Endoplasmic reticulum resident protein 44 | 244 | 8.10e-21 31 | 5,09 | 4.29 | 45 | Endoplasmic reticulum,lumen | QProteome |
| Mitochondrian proteins | | | | | | | | | |
| 85 | Q12931 | Heat shock protein 75 kDa | 143 | 1.00e-10 | 32 | 8.30 | 37 | Mitochondria | QProteome |
| 86 | P10809 | 60 kDa heat shock protein | 521 | 1.60e-48 | 34 | 5.7 | 46 | Mitochondria | QProteome |
| 87 | P47985 | Cytochrome b-c1 complex subunit Rieske | 85 | 6.40e-05 | 9 | 8.55 | 27 | Mitochondria | QProteome |
| 88 | P49411 | Elongation factor Tu | 127 | 4.00e-09 | 19 | 7.26 | 36 | Mitochondria | QProteome |
| 89 | P45880 | Voltage-dependent anion-selective channel protein 2 | 113 | 1.00e-07 | 11 | 7.49 | 43 | Mitochondria, go to nucleus | QProteome |
| 90 | Q13011 | Delta(3,5)-Delta(2,4)-dienoyl-CoA isomerase | 161 | 1.60e-12 | 16 | 8.16 | 30 | Mitochondria, | QProteome |
| 91 | P30084 | Enoyl-CoA hydratase | 225 | 6.40e-19 | 16 | 8.34 | 30 | Mitochondria | QProteome |
| 92 | P25705 | ATP synthase subunit alpha | 78 | 0,00036 | 18 | 9.16 | 25 | Mitochondria | QProteome |
| 93 | O95831 | Apoptosis-inducing factor 1 | 92 | 1.40e-05 | 22 | 9.04 | 31 | Mitochondria,cytoplasim,nucleus | QProteome |
| 94 | P31040 | Succinate dehydrogenase [ubiquinone] flavoprotein subunit | 59 | 0.028 | 17 | 7.06 | 22 | Mitochondrial, | QProteome |
| 95 | P34897 | Serine hydroxymethyltransferase | 64 | 0.0086 | 16 | 8.76 | 22 | Mitochondrial, nucleus | QProteome |
| 96 | P35232 | Prohibitin | 280 | 2.00e-24 | 23 | 5.57 | 56 | Mitochondrial, nucleus, cytoplasm, cell membrane | QProteome |
| Secreted proteins | | | | | | | | | |
| 97 | P07355 | Annexin A2 | 148 | 3.20e-11 | 24 | 7.57 | 53 | Secreted, extracellular space,extracellular matrix, basement membrane | QProteome |
| 98 | P13838 | Spliceosome RNA helicase BAT1 | 79 | 0.00023 | 14 | 5.44 | 32 | Membrane proteins, Mitochondria | QProteome |
| 99 | Q9UJZ1 | Stomatin-like protein 2 | 221 | 1.60e-18 | 35 | 6.88 | 47 | Cell membrane proteins, Mitochondria | QProteome |
| 100 | P15311 | Ezrin | 120 | 2.00e-08 | 33 | 5.94 | 34 | Cell membrane, cytoplasm | QProteome |

***Enrichment of NPs from cultured cells using ReadyPrep (Cytoplasmic/Nuclear) Protein Extraction Kit***

The experiments have been performed as described in the "Methods" section. NPs that were solubilized in a chaotropic buffer were subjected to 2DE separation and 10 randomly selected protein spots were cut-to to identify by MALDI-TOF / TOF analysis. The extent of NPs enrichment was assessed by using a NPs -specifc markers, histonH3, LaminA/C, GAPDH and Cyclophilin A. The intracellular localizations of the identified proteins were determined using SWISS-PROT database or relevant databases. Only two of the identified proteins were localized to nucleus. The rest of the proteins were either cytoplasmic or had no known localization. The repeated experiments generated similar results indicating that, the enriched nuclear protein extracts obtained by this kit was not enriched at a desirable level. Nevertheless, for the sake of verification, enriched nuclear proteins were subjected to WB analysis and four different antibodies were used to visualize the level of enrichment. Histon-H3 and Lamin were used as the nuclear protein markers, and GAPDH and cyclophilin A were used as the cytoplasmic protein markers (Figure 1). WB results showed that NP fractions were partially enriched, and cytoplasmic protein contamination was present at a considerable level. WB results were consistent with the results of 2DE and indicated that the commercial kit only partially enriched nuclear proteins to a desired level.

***Enrichment of NPs from cultured cells using OptiPrep medium***

OptiPrep medium helps isolation of nuclei from cultured cells and allow enrichment of NPs. Experiments with OptiPrep medium was performed as described in the materials and methods section. Proteins in fractions were solubilized in 2D rehydration buffer and were subjected to WB analysis using antibodies against nuclear proteins Histon and, Lamin A / C and the cytoplasmic proteins, GAPDH and Cyclophilin A. The results showed that nuclear proteins were predominantly located in between fractions 24 and 31. However, these fractions were also contaminated with the cytoplasmic proteins (Figure 1). To assess the level of enrichment, 2DE was performed with fraction 29 and spots were randomly cut and identified by MALDI- TOF/TOF analysis (Figure 1) (Table 1).

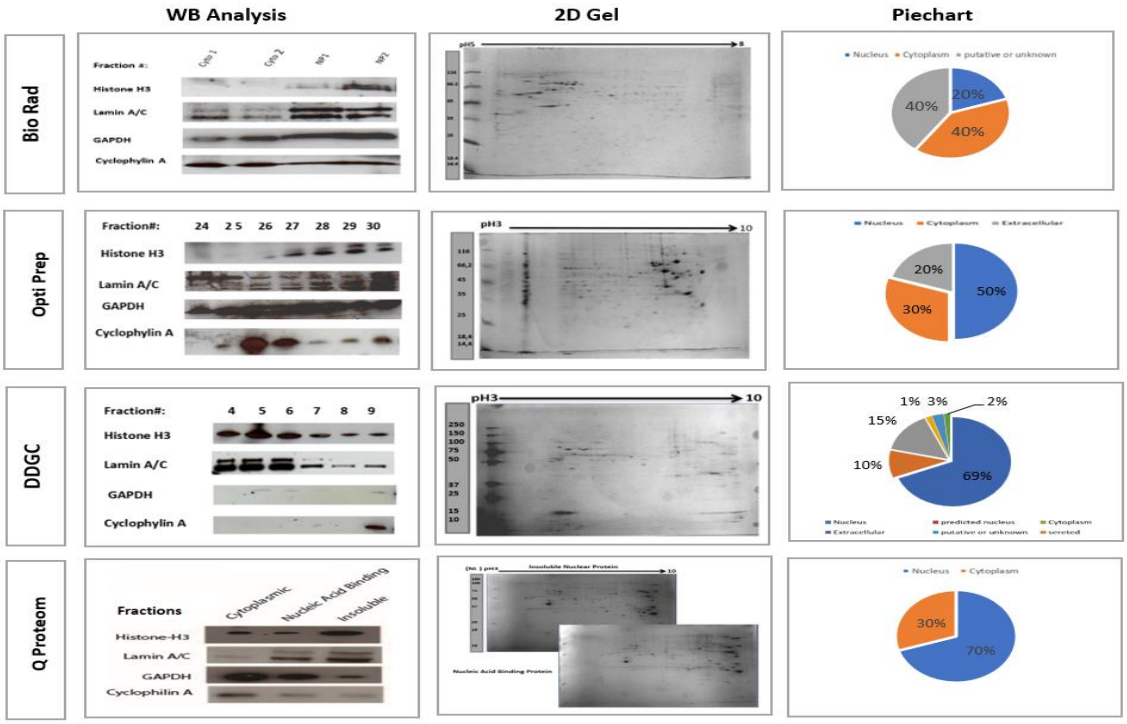


**Figure 1.** Representative images summarizing the major findings of the study. Western Blot analysis of the enriched nuclear protein fractions. Monoclonal antibodies against histone H3 and lamin A/C, GAPDH and Cyclophilin A were used to assess the level of NPs enrichment. Images of 2-DE gels from which the putative NPs were cut and identified. The pie-charts were used to present subcellular localization, BioRad: Extraction of NPs From Cultured Cell using ReadyPrep™ Protein Extraction Kit, OptiPrep: Isolation of nuclei from cultured cell using OptiPrep Application sheet, DDGC: As a combination of methodology 1 and 2, an improved method for extraction of NPs, Qproteome: Qproteome Nuclear Protein enrichment kit.

Ten randomly selected protein spots were cut from a gel and identified by MALDI-TOF / TOF analysis and the intracellular localizations of the identified proteins were determined using SWISS-PROT database or relevant databases. Only two of the identified proteins were localized to nucleus. The rest of the proteins were either cytoplasmic or had no known localization.

The repeated experiments generated similar results indicating that, the enriched nuclear protein extracts obtained by this kit was not enriched at a desirable level. Nevertheless, for the sake of verification, enriched nuclear proteins were subjected to WB analysis and four different antibodies were used to visualize the level of enrichment. Histon-H3 and Lamin were used as the nuclear protein markers, and GAPDH and cyclophilin A were used as the cytoplasmic protein markers (Figure 1). WB results showed that nuclear proteins were partially enriched, and cytoplasmic protein contamination was present in the enriched protein extracts. These results are consistent with the results of 2DE and indicated that the commercial kit failed to enrich nuclear proteins to the desired level.

***Isolation of nuclei from cultured cell using OptiPrep Application sheet***

Extraction of NPs From Cultured Cell using OptiPrep Application sheet was performed as described above mentioned section. Thirty seven fractions were collected to separate proteins based on their molecular densities. The fractions were then subjected to WB analysis using antibodies against Histon, Lamin A / C, GAPDH and Cyclophilin A. "The results showed that nuclear proteins were predominantly located in between fractions 24 and 31. However, these fractions were also contaminated with the cytoplasmic proteins (Figure 1). To assess the level of enrichment, 2DE was performed with fraction 29 and spots were randomly cutted and identified by MALDI-TOF/TOF analysis (Figure 1) (Table 1.)

***Qproteome Nuclear Protein enrichment kit (Qiagen, Stockach, Germany)***

Q-Proteome nuclear protein isolation (Qiagen, USA) was used as an alternative to the differential and density gradient centrifugation approaches. Two different nuclear protein fractions were obtained by following instructions of kit. The first fraction contained the soluble nucleic acid binding proteins, while the second fraction contained the insoluble nuclear proteins. The enriched protein fractions were then subjected to WB analysis using antibodies against Histon, Lamin A / C, GAPDH and Cyclophilin A (Figure 1). The nucleic acid binding and insoluble protein fractions were richer in their histone and Lamin A/C contents. In addition, the cyclophilin A level was much lower than the nuclear fractions indicating that the Q-proteome approach provided satisfactory level of enrichment for nuclear proteins. However, GAPDH was also detected in the enriched nuclear protein fractions, although the level of GAPDH was much lower in comparison to the cytoplasmic fraction. As we stated previously, GAPDH was not a good cytoplasmic protein marker to demonstrate the level of nuclear protein enrichment.

***Enrichment of NPS from cultured cells using discontinues density gradient centrifugation***

As a combination of methodology 1 and 2, an improved method for extraction of NPs was developed. WB analysis revealed the notable presence of histon H3, LaminA/C and even there was no any band signals related to cytoplasmic proteins. To examine the proteomic profiling of enriched proteins, 2-DE analysis was performed. Sixty one spots were identified and classified based on their subcellular location (Table 1). We were able to identify NPs or predicted NPs (69%) and cytoplasmic proteins (15%) along with the proteins belonging to extracellular and unknown or secreted proteins (6%). Overall, improved method approach did not provide sufficient enrichment of NPs despite some success over the other enrichment methods.

The enriched nuclear protein fractions were subjected to 2DE gel electrophoresis to assess the level of enrichment (Figure 1). Spots were randomly cut from the gels and identified by MALDI-TOF/TOF analysis. The results demonstrated that 90 % of the identified proteins in the nucleic acid binding protein fraction were nuclear. Although the insoluble nuclear protein containing fraction had much lower nuclear protein ratio, it was still above 50% indicating that Q-proteome nuclear protein enrichment kit was superior to the other kits/approaches (Figure 2) (Table 1 and 2).

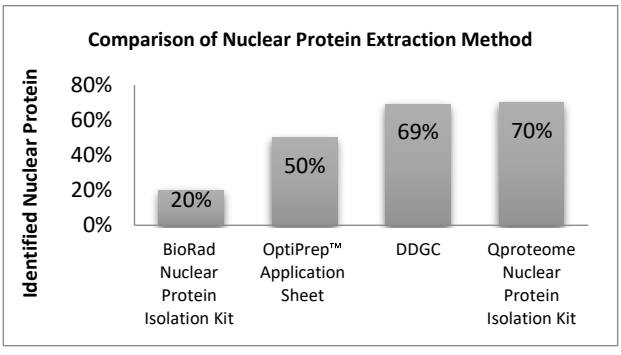


**Figure 2.** Bar graph demonstrating the overall comparison of four different methods for enrichment of NPs.

Comparison of the overall results have indicated that the Q-proteome isolation method was more successful in enrichment of nuclear proteins than the other tested methods (Figure 1). During enrichment of nuclear proteins, two fractions labeled as the nucleic acid binding protein fraction and the nuclear insoluble protein fraction were obtained. Both fractions were subjected to western blot analysis to assess the level of nuclear protein enrichments using nuclear and cytoplasmic protein markers (Figure 1).

Significant increases in the levels of two of the nuclear proteins, histone H3 and lamin A/C, were detected in the enriched nuclear protein fractions, while a significant decrease occurred in the level of cyclophilin A, a cytoplasmic protein. Surprisingly, however, there was no notable change in GAPDH levels in the enriched nuclear protein fractions indicating that GAPDH was not a good enrichment marker for the nuclear protein extracts. On the other hand, there was relatively low level of cyclophilin A in the enriched nuclear protein fractions indicating that the enriched nuclear protein fractions might possess cytoplasmic proteins.

Quantitative evaluation of the level of enrichment was performed by running 2DE gels from which protein spots were cut and identified. Approximately 170 ± 20 protein spots were detected on the gels and 110 of these were excised and identifed by MALDI-TOF/TOF. Cellular localizactions of the identifed proteins were determined using UniProt database. The enriched nuclear protein fractions were subjected to 2DE gel electrophoresis to assess the level of enrichment (Figure 1). Spots were randomly cut from the gels and identified by MALDI-TOF/TOF analysis. The results have demonstrated that 90 % of the identified proteins in the nucleic acid binding protein fraction were nuclear. Although the insoluble nuclear protein containing fraction had much lower nuclear protein ratio, it was still above 50% indicating that Q-proteome nuclear protein enrichment kit was superior to the other kits/approaches (Figure 1) (Table 1 and 2).

## Discussion

As a first sub-cellular organelle discovered in early seventeenth century, nucleus is characterized with a plethora of functions including condensation of chromatin through assemble of long DNA molecules, replication and repair of DNA, gene expression and regulation, synthesis and modification of RNA molecules. Eventhough given their importance, current poor knowledge of high-throughput methods to enrich or extract of nuclear proteins remain limited. Since, this study focused on evaluating of four different enrichment methods to facilitate deeper research of the complex nuclear machinery. There are variety of factors which hampered the enrichment of nuclear proteins. The most striking bottleneck seems to be the contamination of nuclei by cytosolic material during the isolation process.

The classical methods are remain to be used widely due to the advantages of easily adaptation and provide base for more advanced approaches. Here we evaluated four different methods of enrichment of NPs. Based on our evaluation, comparison of the four types of methods results have indicated that the Q-proteome isolation method was more successful in enrichment of nuclear proteins than the other tested methods (Figure 2). Recent developments in methodological and technological advances, new strategies such as FFE, immunopurification, FAOS, LOPIT, PCP and subtractive proteomics were emerged for systematically characterize and isolated various types of subcellular organellar proteomics. For some instances, lately researchers combined subtractive proteomics and proximity-labelling technology coupled with quantitative mass spectrometry to identified about 200 potential candidates for plant NE transmembrane (PNET) proteins from Arabidopsis. Among them, PNET1 is a homologue of human TMEM209, a critical driver for lung cancer which is a bona fide nucleoporin in the plant.[16] In the long run, emerging techniques may be independently or compensation for conventional strategies to obtain organellar proteomics with high-resolution.

**Compliance with Ethical Standards**

Since this study was in vitro cell line study, there was no need for ethical approval.

**Conflict of interest**

The authors declare no conflicts of interest.

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**Author contributions**

AU: Study conception and experimental, Cell culture experiments, Sample preparation and protein isolation, draft of the manuscript; AU, MK: Western Blotting experiments; AU, MK and GA: Two-Dimensional gels electrophoresis, The identification of proteins and image analaysis; MK, GA: Writing, review, and editing. All authors commented and approved the final version of the manuscript.

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