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Efficient sequential recovery of nucleolar macromolecular components

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Abstract

Efficient extraction and accurate quantification of nucleolar macromolecules are critical for in vitro analysis, especially for studying RNA, DNA, and protein dynamics under identical conditions. There is presently no single method that efficiently and simultaneously isolates these three macromolecular constituents from purified nucleoli. We have developed an optimized method, which without evident loss, extracts, and solubilizes protein recovered from a single sample following TRIzol isolation of RNA and DNA. The solubilized protein can be accurately quantified by protein bicinchoninic acid assay and assessed by polyacrylamide gel electrophoresis. We have successfully applied this approach to extract and quantify all three nucleolar components, and to study nucleolar protein responses after actinomycin D treatment.

Keywords

Cell biology; DNA; Macromolecular; Nucleolus; RNA; TRIzol

The nucleolus is a nonmembrane bound structure composed of ribosomal (r) DNA, RNA, and protein. The nucleolus is not only involved in ribosome production, but also participates in aspects of RNA processing, as well as in the regulation of mitosis, cell growth, and programmed cell death [1–3]. Several studies have shown that the nucleolus undergoes gross morphological changes in response to various cellular stresses, and have presented quantitative data indicating that these changes are associated with the selective reorganization of the nucleolar proteome [4, 5]. In order to study the effects on the proteome by in vitro analyses such as gel electrophoresis and immunological detection, efficient extraction, and accurate quantification of proteins from the purified organelle are critical.

Although nucleoli can be successfully separated from the cells, most buffers used for protein extraction (e.g. RIPA: Tris-HCl buffer containing 1% NP-40 and 1% SDS) are not efficient [6]. To maximize the recovery of nucleolar protein, nucleoli have often been directly lysed in denaturing gel loading buffer [7, 8], which is not compatible with protein quantification assays. Much effort has been put into increasing the recovery of protein from the nucleolus, and various procedures and methodological combinations have been tested. For example, sonication of purified nucleoli in RIPA buffer only slightly increases the protein extraction efficiency compared with RIPA buffer only [6]. DNase treatment has been used to release nucleolar proteins bound to rDNA [6], and high salt buffer to precipitate specific proteins

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[9]. Overall, each of these techniques has offered limited improvements. Moreover, most studies have focused on only one aspect of nucleolar biomolecules (protein, RNA, or DNA), and till now there has been no single method to study the changes in the nucleolar macromolecular components simultaneously and under comparative conditions.

Here, we present a TRIzol-based method that efficiently and sequentially releases RNA, DNA, and protein components from cellular preparations (Fig. 1A). TRIzol is a robust lysis and denaturing reagent, which is primarily designed for RNA extraction, and which has generally not been used for the extraction of DNA and protein. One reason for this is that TRIzol-extracted proteins are difficult to dissolve using the standard solubilizing reagents recommended by the TRIzol user manual (Invitrogen, CA, USA) [10]. To overcome this problem, we have developed a buffer that efficiently dissolves nucleolar proteins precipitated after TRIzol lysis. The buffer is compatible with the Pierce (bicinchoninic acid (BCA) protein assay.

To assess for conditions for improved protein recovery following TRIzol extraction, human epithelial carcinoma (HeLa) cells were directly lysed in TRIzol reagent, and the RNA, DNA, and protein components were isolated as depicted in Fig. 1A. Following the separation of RNA and DNA, isopropyl alcohol was added and the insoluble pellet was collected following centrifugation as recommended in the manufacturer's protocol. The pellet was dissolved into 50 μL buffer with increasing concentrations of urea up to 4.5 M in the presence of 0.5% SDS at room temperature (RT). After 1 h, the samples were centrifuged at 12 000 × *g* for 10 min at RT. The supernatants were collected, 1× Laemmli sample buffer (LSB) was added, boiled, and an equal volume of each (10 μL) was loaded into SDS-PAGE. High urea concentrations increased the solubility as shown by CBB staining (Fig. 1B), and protein quantification of the supernatants using BCA assay (Fig. 1C). Neither SDS nor urea alone could completely dissolve the precipitated protein (Fig. 1B, lanes 7 and 8). To control compatibility of the BCA assay with 4.5 M urea, BSA was dissolved in the SDS/urea buffer. The analysis showed linear performance in the BCA protein assay (Fig. 1D). For subsequent experiments, we used 4.5 M urea in 0.5% SDS, 25 mM Tris/HCl pH 7.5, and labeled this buffer as SUBT.

Most buffers for protein extraction are designed to extract soluble protein by employing various detergents (e.g. NP-40 or Triton X-100). However, TRIzol lyses the entire cellular organelle [11] and subsequently precipitates both soluble and insoluble protein, which may then be recovered from the precipitate with the SUTB buffer. To test the efficiency of the SUTB buffer in this respect, we compared the recovery of proteins from the TRIzol based method to two other regularly used methods for extraction of both soluble and insoluble proteins. HeLa cells were harvested and divided into three equal aliquots. Equal volume of each extraction buffer (50 μL) was subsequently used. One aliquot was extracted with TRIzol and the protein was solubilized in SUTB. The two other aliquots were lysed either in UTB (9 M urea, 75 mM Tris/HCl pH 7.5, and 0.15 M β-mercaptoethanol) or cell disruption buffer (PARIS kit, Ambion, TX, USA). Loading of equal volumes of extracted protein (8 or 16 μL corresponding to approximately 10 or 20 μg, respectively) on an SDS-PAGE followed by staining with CBB showed that the concentration and composition of the recovered protein was almost identical with the three methods (Fig. 1E). The results thus indicate that protein redissolved from TRIzol extraction were comparable with other extraction methods that perform well in cell solubilization assays.

To demonstrate the practical applicability of TRIzol in combination with SUBT for simultaneous analysis of DNA, RNA, and protein from the nucleolus, we purified nucleoli, cytoplasm, and nuclei from HeLa cells according to protocols from the Lamond lab (http://www.lamondlab.com/f7nucleolarprotocol.htm.) (Fig. 2A). After separation of the nucleolar,

nuclear, and cytoplasmic fractions, RNA was first extracted with TRIzol from each fraction. To ensure complete dissolution of the nucleoli, the nucleoli were vortexed in TRIzol until homogeneous, followed by standard RNA purification. Equal amounts of total RNA (5 μg) from the three cellular compartments were separated on 1.2% glyoxal agarose gel (Fig. 2B, left panel). The major RNA forms in the nucleolar fraction were precursor rRNA (47S) and processed rRNA intermediates (36/32S). The 28S and 18S rRNAs are rapidly transported from the nucleolus [12] and are thus less abundant in this compartment, while being clearly detectable in both nucleus and cytoplasm. We further performed Northern blotting of the RNA using a probe targeting the 5′ ETS region of the 47S precursor rRNA (Fig. 2B, right panel).

DNA was then purified from the subcellular fractions. The integrity of nucleolar DNA was tested by digestion with HindIII for 2 h and separated on 0.8% agarose gel compared with the DNA sample without digestion. The undigested DNA remained in the loading well, suggesting that the TRIzol-extracted DNA was mainly high-molecular weight DNA (Fig. 2C). The integrity of the TRIzol-purified nuclear and nucleolar DNA was further tested by PCR amplification of two genomic DNA regions (rDNA and GAPDH locus) of at least 1 kbp length. As shown in Fig. 2D, both products were successfully amplified.

Finally, the insoluble protein samples obtained with TRI-zol extraction of nucleolar, nuclear, and cytoplasmic specimens were dissolved in SUTB, and quantified using the BCA protein assay. Equal amounts of protein (15 μg) from each sample was loaded on a 3–8% Tris-acetate NuPage protein gel and stained with CBB. The proteins were well separated, and the nucleolar proteins were distinctly different from those in the other cellular compartments (Fig. 3A). Overall, approximately 400 μg of protein was repeatedly recovered from the nucleolar specimens of 4×10^8 HeLa cells, and had an excellent correlation to the amount of RNA isolated in four independent experiments (Fig. 3B). To demonstrate that the TRIzol-based method can recover nucleolar proteins without significant loss, we compared this with two other most common extraction methods. For these purposes, purified nucleoli were divided into three equal aliquots. One aliquot was directly dissolved in LSB. To reduce viscosity, the sample was passed through a QIAshredder column to break genomic DNA. A second aliquot was extracted with TRIzol and solubilized in SUTB. The SUTB sample was centrifuged at 12 000 × *g* for 10 min and the supernatant was collected (SUTB-S). 1× LSB was added to the pellet fraction (SUTB-P). The third aliquot was extracted using RIPA buffer followed by centrifuging to separate supernatant from the pellet, and the pellet was dissolved in 1× LSB buffer and passed through QIAshedder column (RIPA-P). All samples were boiled and loaded on a Novex 3–8% TAE gel to separate proteins with molecular weights above 35 kDa (Fig. 3C, left panel) or on Novex 4–12% Bis-Tris gel to separate low molecular weight proteins (Fig. 3C, right panel). As evident in both gels, the supernatant from SUTB had a protein composition that was almost identical to that of the sample directly lysed in LSB. No protein was detectable in the SUTB pellet, suggesting that SUTB efficiently solubilizes nucleolar protein. In contrast, the recovery of protein following RIPA extraction was poor, with 55% of protein remaining in the insoluble pellet fraction (Fig. 3D).

To confirm that SUTB-solubilized protein samples are suitable for immunoblotting, proteins from the three nucleolar subcompartments were probed with specific antibodies against each (Fig. 3E). Proteins with molecular weights ranging from 38 to 194 kDa and representing fibrillar (RPA194, UBF) and dense fibrillar center (FBL) and granular component (NPM) were easily detectable in the nucleolus (Fig. 3E). As a further demonstration of the practical application of the method, we studied the nucleolar response to actinomycin D (actD) treatment (see ref. [4] for comparison). Subcellular fractionation was conducted on mock and actD-treated HeLa cells. The proteins were redissolved in SUTB. Western blot was carried out against fibrillarin (FBL) and nucleolin (NCL). As expected [4], the nucleolar

concentration of NCL was decreased by the actD treatment, while the FBL concentration remained constant (Fig. 3F).

We have shown that the TRIzol reagent can be effectively used to extract not only RNA, but also DNA and protein from the nucleolus. We show that the SUTB buffer can fully solubilize TRIzol-extracted nucleolar protein without any evident loss. The SUTB buffer is compatible with protein quantification assays, which is a substantial advantage toward estimating protein yield and comparison of proteomic changes under different experimental conditions. The protein specimens are compatible with several detection methods, including mass spectrometry [13]. However, the high urea concentration is likely to be prohibitive to protein–protein interaction analyses, although this might be partially overcome using dialysis. Thus, the ability to re-dissolve precipitated protein after TRI-zol extraction allows for direct comparison of genomic, transcriptomic, and proteomic features from the same sample, which is extremely useful when available material is limited as e.g. for nucleolar samples, and separate preparations for nucleic acid and protein extractions may not be feasible.

Abbreviations

5′ETS	5′ external transcribed spacer
actD	actinomycin D
BCA	bicinchoninic acid
Cyto	cytoplasmic
GAPDH	glyceraldehyde 3-phosphate dehydrogenase
HeLa	human epithelial carcinoma
LSB	Laemmli sample buffer
No	nucleolus
Nu	nucleus
r	ribosomal
RT	room temperature

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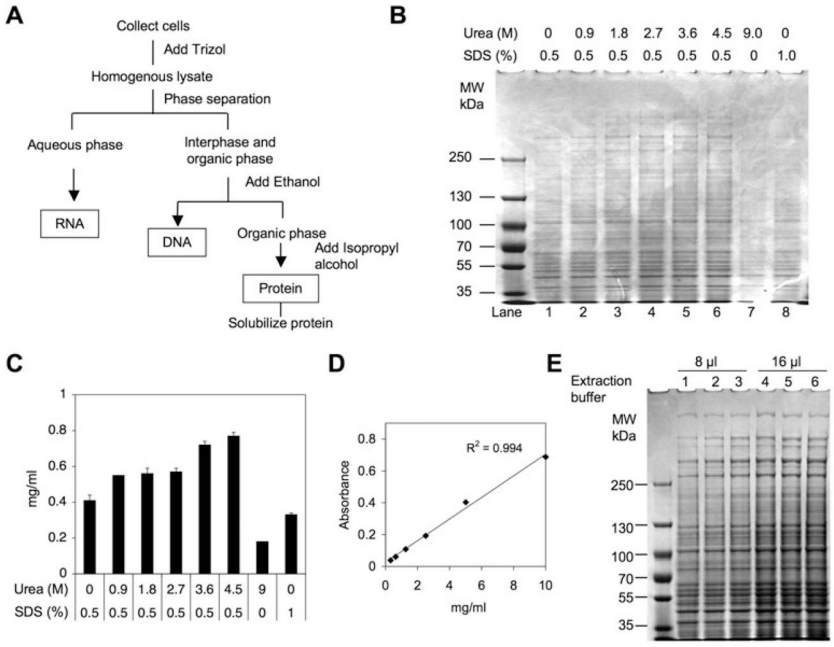

Figure 1.

Analysis of TRIzol-extracted total cellular protein. (A) Diagram of separation of RNA, DNA, and protein using the advanced TRIzol protocol. (B) Effects of urea and SDS on the solubility of TRIzol-extracted protein, as evaluated by SDS-PAGE and stained by CBB. The protein pellets were dissolved with the indicated concentrations of urea and SDS in a volume of 50 μL, followed by centrifugation. Supernatants were collected, LSB was added and 10 μL of each sample was loaded to the gel. The experiment was repeated two times with comparable results. (C) Protein quantification by the BCA protein assay. Samples are as in (B). Error bars, SD; $N = 2$. (D) Standard curve of BSA dissolved in SUTB. SUTB buffer is compatible with protein quantification using BCA. Linearity of the assay is shown by Pearson correlation analysis (R^2). (E) Extraction of total cellular protein using three different buffers. Each sample represents cell supernatants from an equal amount of cells. Lanes 1 and 4, TRIzol; lanes 2 and 5: SUTB, lanes 3 and 6: Ambion cell lysis buffer. Eight microliters of protein sample correspond to approximately 10-μg protein.

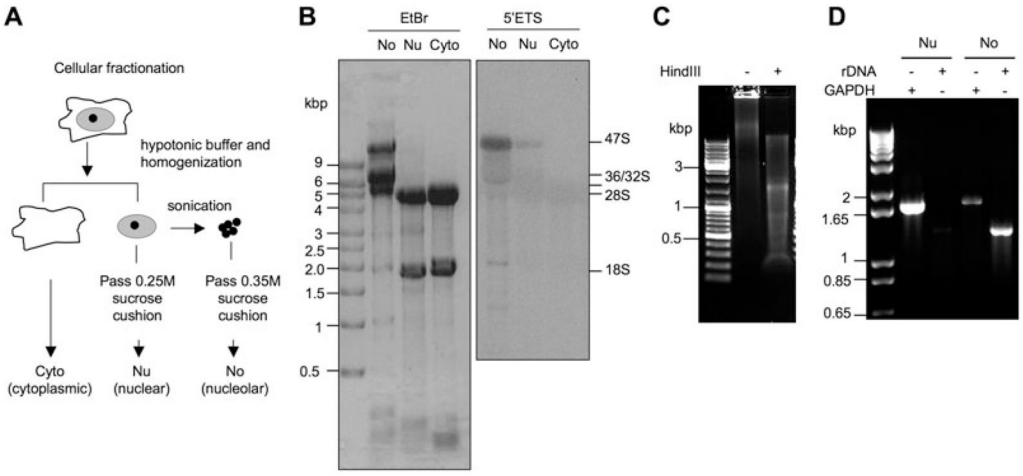

Figure 2.

TRIzol extraction of RNA and DNA from cellular subfractions. (A) Procedure of cellular fractionation to purify cytoplasmic (Cyto), nuclear (Nu), and nucleolar (No) fraction. Note that the nuclear fraction contains also the nucleoli. (B) RNA isolated from No, Nu, and Cyto (5 μg each) was analyzed by 1.2% glyoxal agarose gel, and stained with EtBr (left panel). N = 5. A representative image is shown. The same gel was transferred to nylon membrane, followed 5′ external transcribed spacer (5′ ETS) detection using Northern hybridization (right panel). 5′ETS is only present in the 47S precursor rRNA, and is degraded during rRNA processing in the nucleolus. Mature and cleaved forms of rRNA are indicated to the right. (C) DNA was isolated from the nucleolus using TRIzol, digested with or without HindIII and separated on a 0.8% agarose gel. (D) PCR amplification of long regions (>1 kbp) of DNA extracted using TRIzol from Nu and No. DNA fragments corresponding to rDNA and glyceraldehyde 3-phosphate dehydrogenase (GAPDH) locus were amplified as indicated. PCR products were assessed by 1.2% agarose gel.

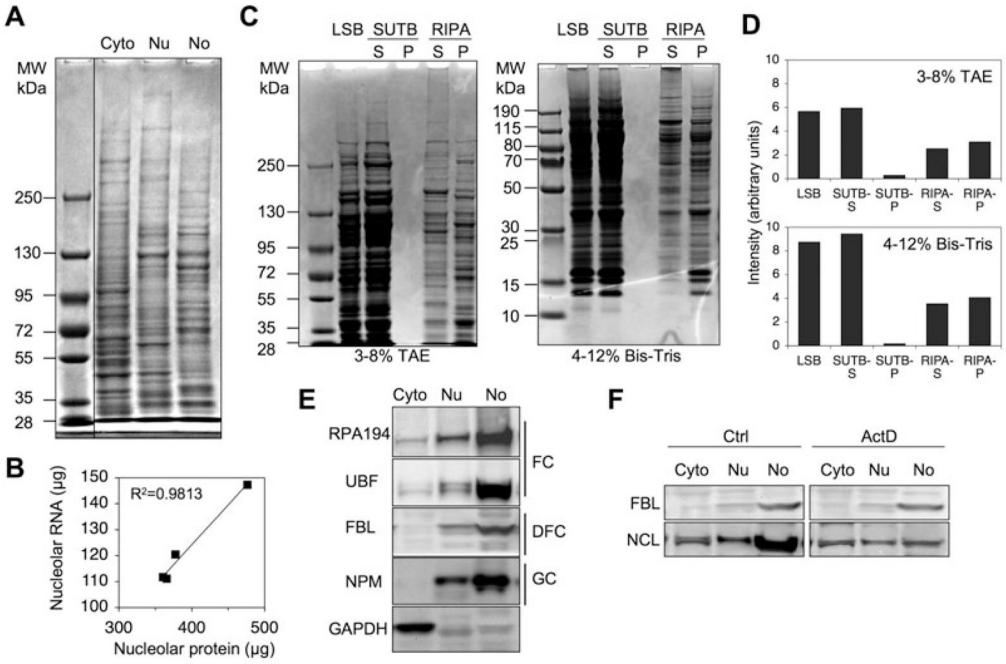

Figure 3.

Characterization of TRIzol-extracted proteins from cellular subfractions. (A) Proteins from the nucleolar No, Nu, and Cyto fractions were separated on 3–8% Tris-Acetate Nu-PAGE and stained by CBB. Fifteen micrograms of protein was loaded of each fraction. M, molecular weight markers. $N = 5$ independent experiments. A representative image is shown. (B) Correlation of the recovery of nucleolar protein and RNA from four independent preparations of HeLa cells (~400 $\times 10^6$ cells each). $N = 4$. (C) Comparative extraction of nucleolar proteins using three different methods. The following extractions were used: 1× LSB, SUTB solubilization (SUTB), or RIPA lysis buffer. All extractions were based on equal numbers of isolated nucleoli. S, soluble supernatant; P, insoluble pellet. The samples were separated on 3–8% TAE (left panel) or 4–12% Bis-Tris (right panel) gels and stained by CBB. (D) Quantification of CBB stained proteins in (C) using densitometry. (E) Western blot analysis of SUTB-solubilized nucleolar (Nu), cytoplasmic (Cyto), and nuclear (Nu) proteins. Proteins representative of three subnucleolar compartments, the fibrillar centre (FC) RPA194 and UBF, the dense fibrillar centre (DFC) fibrillarin (FBL), and the granular component (GC) nucleophosmin (NPM) are shown. GAPDH was used as a cytoplasmic marker. Forty micrograms of protein was loaded. $N = 2$. (F) Western blot analysis of FBL and NCL of cells treated with actinomycin D (50 ng/mL) for 3 h. Forty micrograms of protein was loaded.