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Improved reconstitution of Trizol derived protein extracts provides high quality samples for comprehensive proteomic characterization of cell cultures

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ABSTRACT

Background: The study of RNA, DNA, and protein from the same sample is a great advantage but can be challenging. Using Trizol, one can simultaneously extract RNA, DNA, and protein, leading to efficient sample use and more comprehensive analysis. Although it is used routinely for RNA extraction, the frequency of use of Trizol extracts for proteomics applications is low. The aim of our study was to evaluate the results of a simple modification to the Trizol protocol in terms of extraction and protein recovery efficacy and compatibility of the extracts with proteomics technologies in comparison to our standard extraction protocol including freeze/thaw cycles in urea/ thiourea. *Method:* We used the human airway epithelial cell line S9 and extracted proteins either with a modified Trizol protocol or by freeze/thaw cycles in 8M urea/ 2M thiourea. Extracted proteins were quantified and subjected to 1D- and 2D-gel electrophoresis, Western Blotting and LC-coupled tandem mass spectrometry analysis. *Results:* Compared to urea/ thiourea extraction, the Trizol-extracted proteins exhibited a similar protein composition and identification rate in LC-coupled tandem mass spectrometry experiments. 1D- and 2D-PAGE of Trizol-extracted proteins revealed excellent protein resolution with better coverage of proteins in the low MW range than urea/ thiourea extraction. *Conclusion:* The modified Trizol-protocol enabled excellent protein extraction from cell culture samples and high compatibility with proteomics technologies, especially with LC-tandem mass spectrometry.

Keywords: Trizol; cell culture; nano-HPLC-MS/MS; 1D PAGE; 2D PAGE; Western Blot Analysis.

Abbreviations

1D: one dimensional; **2D:** two dimensional; **DTT:** dithiothreitol; **ESI:** electro spray ionization; **FTICR:** fourier transform ion cyclotron resonance; **GAPDH:** glyceraldehyde 3-phosphate dehydrogenase; **HPLC:** high performance liquid chromatography; **LTQ:** linear ion trap; **MS:** mass spectrometry; **MS/MS:** tandem mass spectrometry; **PSMs:** peptide spectrum matches; **SDS:** sodium dodecyl sulfate; **TBS-T:** Tris-buffered saline-Tween; **UT:** urea/ thiourea ; **AP-NBT:** alkaline phosphatase-nitrotetrazolium blue/5-bromo-4-chloro-3-indolyl-phosphate.

1. Introduction

In-depth physiology and pathophysiology studies profit from complementing analyses of gene expression and protein abundance patterns of various conditions [1]. While transcriptomics provides comprehensive information on gene expression, proteomics allows direct analysis of protein levels accounting for regulation of protein stability and post translational modifications as well [2]. However, both techniques are complementing, suggesting that combined analyses offer a much more comprehensive view of the physiology or pathophysiology of the sample at the molecular level [3]. For practical reasons, RNA and protein extraction from the same sample would be ideal, ensuring the same

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physiological state to be reviewed. Trizol (Invitrogen) or Qiazol (Qiagen) are commercial solutions of ready-to-use reagents containing a monophasic solution of phenol, guanidine isothiocyanate and chloroform to isolate nucleic acids and protein from different biological sources in a multiple-step method from the same biological sample [4,5]. Trizol is being used primarily and successfully for RNA extraction and analysis because it acts as RNA stabilizing agent by non-specifically denaturing proteins and disrupting enzyme activity, including RNases, thus also yielding low abundance and labile mRNAs [6,7]. Trizol reagent is not yet routinely used, when protein profiling is the main aim of a study, mainly due to difficulties with the resolubilization of the precipitated protein [8]. Modified Trizol protocols that improve the resolubilization of proteins have been developed [8–11]. They have been applied to various protein sources for different proteomics applications. However, as they use detergents such as CHAPS or SDS, they are compatible with 2D-PAGE but not with LC-tandem mass spectrometry.

In the present study we compare a modified Trizol protocol with urea/ thiourea protein extraction applied to the extraction and solubilization of proteins from a human airway epithelial cell line as a proof-of-principle study. We demonstrate the efficacy of the Trizol-protocol for protein extraction and the high compatibility of the extracted proteins with proteomics methods like 1D- and 2D-PAGE, Western Blotting and especially ESI-LC-tandem mass spectrometry.

2. Material and Methods

2.1. Biological samples

The biological model used was the adeno12-SV40-immortalized human airway epithelial cell line S9 (ATCC® number CRL-2778) cultured in an adapted minimal essential medium (MEM; PromoCell, Heidelberg, Germany) as described earlier [12]. Cells were cultured in six culture plates and independently harvested at a cell density of approximately 5x106 cells. All subsequent processing steps and experiments were performed independently for each sample to be able to judge overall variation of the procedure. Three samples were used for Trizol (Invitrogen, Darmstadt, Germany) protein extraction (T1-3), while the other three samples (UT1-3) were lysed in urea/ thiourea as described earlier [13].

2.2. Protein extraction and quantification

2.2.1. UT protein extraction protocol

Cell culture samples were lysed separately in 1000µl 8M urea/ 2M thiourea (UT) by subjecting them to 5 cycles of freezing in liquid nitrogen and subsequent shaking (1500 rpm; 10 min; 37oC). Afterwards, high molecular weight nucleic acids were fragmented by sonication on ice three times for 3s, each with nine cycles at 80% energy using a Sonoplus (Bandelin, Berlin, Germany). Cell debris was removed by centrifugation (21000 g; 30 min; 4^{0}C) and the supernatant was collected for further analyses [13].

2.2.2. Modified Trizol protocol

Protein extraction with Trizol reagent (Invitrogen) was performed according to the manufacturer's protocol, with a particular modification in the reconstitution of the protein pellet, as previously described for protein extraction from heart biopsies [14]. In detail as illustrated in Fig. 1, samples were homogenized and cells were disrupted by pipetting up and down after adding 700 µl Trizol in each sample. Next, samples were incubated (room temperature; 5 min), chloroform was added, the vials were centrifuged (12000 g; 15 min; 4^{0}C) and the resulting upper aqueous phase, containing RNA, was aspirated and stored. DNA was precipitated by adding 210 µl 100% ethanol and sedimented by centrifugation (2000 g; 15 min; 4^{0}C). The supernatant was collected and 100% isopropanol was added for protein precipitation. After incubation (room temperature; 10 min), samples were centrifuged (12000 g; 10 min; 4^{0}C) and the supernatant was discarded. The resulting protein pellets were washed three times with 0.3M guanidine hydrochloride in 95% ethanol, each step being followed by centrifugation (7500 g; 5 min; 4^{0}C). Finally, protein pellets were washed with 100% ethanol and left to air dry for 5-10 min, carefully avoiding extensive (over-)drying. To ensure maximum protein reconstitution, 400 µl UT was added to each sample with multiple dispensing/aspirating cycles, followed by shaking (800 rpm; 20-40 min; 20^{0}C). Samples were stored at -80^{0}C until further use.

A Bradford assay kit (Pierce, Thermo Scientific, Bonn, Germany) and bovine serum albumin as standard protein [15] were used for determination of protein concentration in the samples.

2.3. 1D SDS-PAGE and Western Blotting

Protein samples (20µg) were resolved on 12.5% SDS (sodium dodecyl sulphate) polyacrylamide gels and the patterns were visualized by staining with Coomassie brilliant blue R-250. For specific protein detection (Western Blot analysis), proteins were transferred from the gel onto a 0.45µm pore diameter PVDF (polyvinilidene fluoride) membrane using a semidry Milliblot apparatus (Merck Millipore, Billerica, MA, USA). To control blotting efficiency on the membrane, proteins were visualized using ink in 1% acetic acid and TBS-T (Tris-buffered saline-Tween containing 20mM Tris-HCl, 137 mM NaCl and 0.1% Tween-20). After visualization, ink was removed with TBS-T, the membrane was blocked with 5% powdered milk for 90 min and incubated with the primary antibody mouse anti-α-GAPDH (Cell Signaling Technology, Boston, Massachusetts, USA; dilution 1:50000) over night at 4^{0}C. Detection was

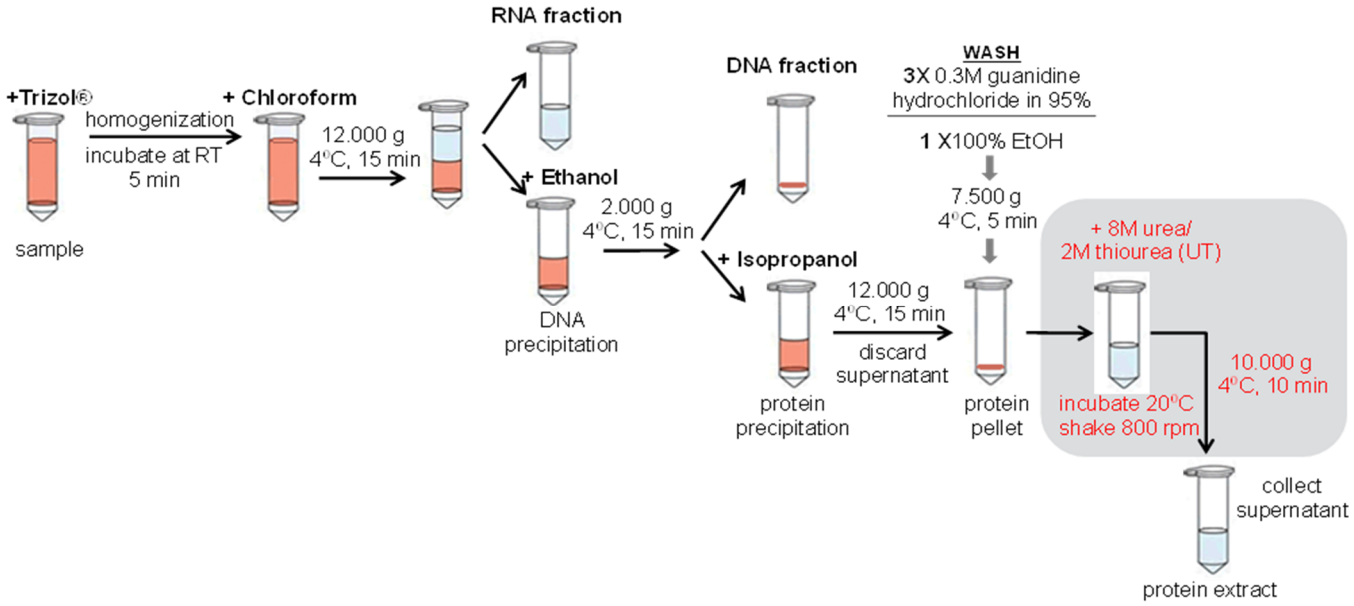

Fig. 1 Modified Trizol protocol for mass spectrometry-compatible protein extraction. The protocol retains the steps suggested by the manufacturer for preparation of RNA-and DNA-fractions, but uses modified steps for an improved reconstitution of the protein pellet in 8M urea/2M thiourea (UT). The modification is highlighted by blue background.

performed after incubation with alkaline phosphatase conjugated goat anti-rabbit IgG (Biorad, Munich, Germany, 1:5000) as secondary antibody for 60 min using the AP-NBT/BCIP in situ detection system.

2.4. 2D SDS-PAGE

Isoelectric focusing was performed using 7cm immobilized pH gradient (IPG) strips (Bio-Rad) with a pI range of 3-10. Strips were loaded with 30 µg proteins in rehydration buffer (8M urea, 2M thiourea, 2% CHAPS, 30mM DTT, 2% pharmalyte and bromophenol blue) and subjected to isoelectric focusing. As described previously [16], equilibration buffers were used for reduction and alkylation of proteins on the strips. Proteins were separated in the second dimension as previously described [17] on 12.5% SDS polyacrylamide gels in low fluorescent glass plates. Finally, Coomassie brilliant blue R-250 staining was used for protein spots visualization.

2.5 Mass spectrometry analysis

Mass spectrometry analysis was performed on three biological replicates for each extraction method (UT and Trizol), using the LTQ-FTICR mass spectrometer (Thermo Scientific, Bremen, Germany) after pre-fractionation of peptides by reverse phase nano-UPLC (Waters, Manchester, U.K.). In total, 4µg protein from each sample was first reduced (2.5mM dithiothreitol; 1 h; 60°C), then alkylated (10 mM iodoacetamide; 30 min; 37°C) and subsequently digested with trypsin (Promega, Madison, USA) in a 1:10 ratio (overnight – 16h; 37°C) as previously described [16]. Proteins were identified using the SEQUEST algorithm with Proteome discoverer 1.3 (Thermo Scientific). MS spectra were searched against a UniProt Swiss-Prot database (rel. 2010_11) limited to human entries with a mass tolerance of 10 ppm for peptide identifications and 0.6 Da fragment tolerance. Methionine oxidation was set as variable, carbamidomethylation at cysteine as fixed modification and up to two missed tryptic cleavages were considered (for details see Supplemental Table S1).

3. Results and discussion

Various modifications of the original Trizol protocol have been reported (Table 1). The majority of them implemented detergents in order to improve protein recovery efficiency for subsequent global protein profiling by 2D-PAGE. Predominantly detergents like CHAPS or SDS were considered to improve protein reconstitution [9,10,18–21]. However, such detergents are incompatible with the nano-HPLC coupled ESI-LC tandem mass spectrometry, except when particular sample clean-up steps are applied. Therefore, here we present the results of a simple modification of the Trizol protocol avoiding the use of detergents but enhancing protein recovery by reconstituting the protein pellet not only in the denaturing chemical urea (8M) but in the presence of 2M thiourea which especially supports the resolubilization of hydrophobic proteins [22]. Thiourea is a non-chaotropic compound, which has been frequently used in 2D-PAGE applications due to its high capacity to re-solubilize membrane proteins [20,22]. Thus, addition of UT followed by incubation at room temperature and shaking at 800 rpm resulted in a rapid and almost

Table 1 Published Trizol protocols modified with the aim of improving protein recovery.

Re-suspension of protein pellets in :	Author	Ref.
1:1 solution of 1% SDS and 8 M urea in Tris-HCl 1 M, pH 8.0, followed by 5 cycles of 15 sec sonication and 30 sec ice incubation	Simões et al.	[10]
7 M urea, 2 M thiourea, 4% (w/v) Chaps, 1 mM phenylmethane sulfonyl fluoride and 30 mM Tris-HCl, pH 8.5	Yamaguchi et al.	[20]
8 M urea, 4% (w/v) Chaps and 2% (w/v) DTT, followed by sonication (10 min, 4°C) and incubation at room temperature for 2 h	Xiong et al.	[19]
1% SDS, followed by incubation at 50°C for longer than 10 min with intermittent vortexing	Likhite et al.	[9]
8 M urea	Ham et al.	[21]
9.5 M urea and 2% (w/v) Chaps, pH 9.1 or 10% acetonitrile, pH 4.8 or 1% triton, pH 5.3	Man et al.	[11]
250 mM glycerol, 10 mM triethanolamine and 4% (w/v) Chaps	Kirkland et al.	[18]
Sonication in methanol and reconstitution of the powder in 0.2% Rapigest	Kline et al.	[26]

complete dissolution of the protein pellet. Due to the strong denaturing conditions used, proteins lose their native conformation and cannot be used for studies of natural activity. However, they are well suited for proteomics studies, including protein quantitation.

3.1 Protein extraction, reconstitution and quantitation

Standard extraction of proteins from 5 x 10^6 S9 cells was performed with 1000µl UT. In contrast, 400 µl UT were used for the reconstitution of the pellet obtained by precipitation of protein with Trizol. Due to the lower volume, protein concentrations of the Trizol derived protein extracts were similar to UT protein extraction (T1=1.75µg/µl, T2=1.37µg/µl, T3=0.67µg/µl; UT1=1.15µg/µl, UT2=0.98µg/µl, UT3=2.32 µg/µl). The total amount of protein extracted with UT was larger compared to Trizol extraction (T1=698.2µg, T2=547.32µg, T3=268.11µg and UT1=1150µg, UT2=983µg, UT3=2324.85µg). Lower protein yield with Trizol protein extraction was also previously reported [20]. However both extraction methods yielded sufficient protein for further proteomics and biochemical analysis methods.

3.2 Resolution and antigenic stability testing of the proteins extracted

Separation of all protein extracts on a 1D-gel revealed a similar complex protein pattern for both extraction methods (Fig. 1A). Minor differences were only observed in the staining intensity of particular protein bands in the low molecular weight (MW) range. Previous reports indicated that Trizol extraction might be more efficient for proteins with low-MW in comparison to other methods of extraction [20,23]. In order to assess if higher amounts of low MW proteins are indeed accessible after Trizol extraction or if the increased band intensity (Fig. 2) resulted from degradation of high MW proteins, the low MW regions were cut from the gel and subjected to in-gel-tryptic digestion and mass spectrometry. However, no differences in the spectral counts per protein between the UT and Trizol derived protein extracts and no indication of increased levels of degradation products were found. Thus, the stronger intensity of bands of low MW proteins after Trizol extraction is likely caused

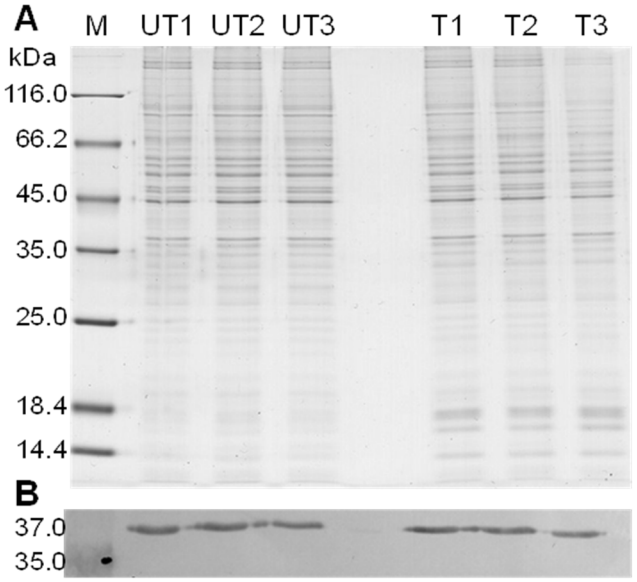

Fig. 2 (A) Visualization of UT and Trizol extracted proteins on a 1D 12.5% SDS polyacrylamide gel by Coomassie staining; (B) Determination of the antigenic stability of the extracted proteins through Western Blotting for the presence of α-GAPDH (at 37kDa). (M-protein marker)

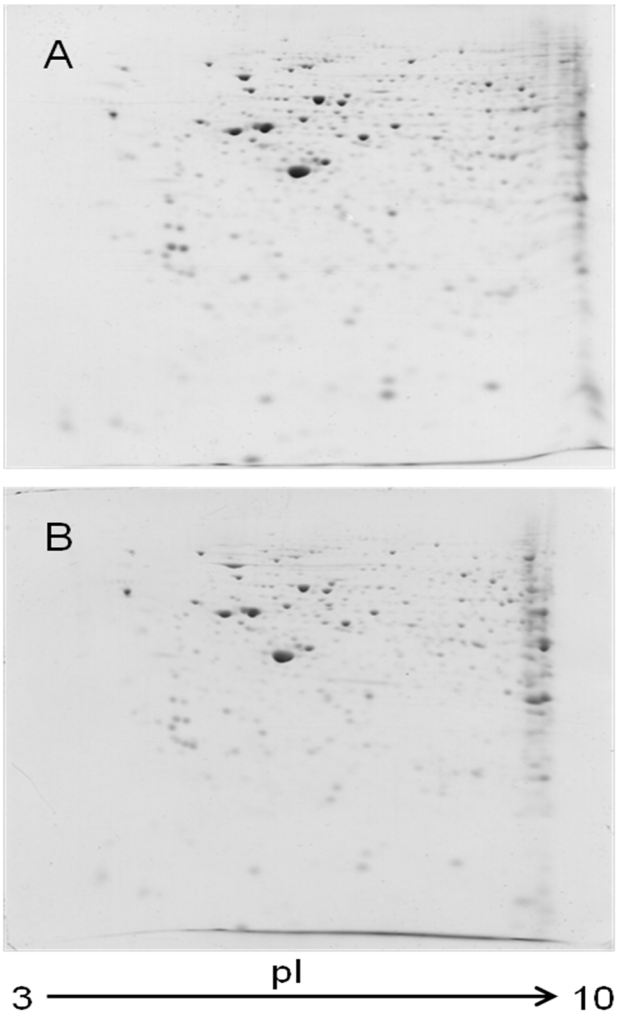

Fig. 3 Representative 2D-PAGE gel images of the proteins extracted using (A) Trizol and (B) UT which show similar protein patterns, good resolution of proteins, and more spots, with a higher intensity, especially in the low MW range for the Trizol-extracted proteins.

by more efficient protein staining.

Since an artificial Trizol-triggered modification of proteins that might lead to a masking of epitopes has previously been discussed [19], the suitability of Trizol-prepared protein extracts for immunoblotting was exemplarily tested for α-GAPDH. Fig. 1B shows α-GAPDH identified at approximately 37kDa, demonstrating preservation of epitope recognition of the proteins extracted and the lack of influence of the extraction method on α-GAPDH signal intensity. Other studies have also investigated the stability of the proteins extracted with Trizol by using PAGE and Western blot analysis, with similar results [8,9,20].

Representative gels of 2D-gel electrophoresis performed with Trizol and UT protein extracts from S9 cell line are presented in Fig. 3. Our modified Trizol extraction method revealed good resolution of proteins, presenting a similar protein pattern as it was detected on the gels in which proteins of the UT extracts were separated. Thus, our data contradict those of Xiong and colleagues, who reported spot chains, smears or diffuse spots or even potential protein degradation as possible consequences of artificial protein modifications in the presence of Trizol [19]. In agreement with our data, comparable good results obtained after conventional urea extraction were reported for 2D-PAGE experiments with Trizol derived extracts [20]. Again, 2D gels of Trizol extracts yielded more spots, with a higher intensity, especially in the low MW range. This effect was hypothesized to occur as a result of the high efficiency of removal of nucleic acids, lipids, carbohydrates and salts [18]. Such a high purity of the protein extracts can be a significant advantage for different protein enrichment methods such as immobilized metal affinity chromatography (IMAC) for phosphopeptides, potentially providing an increased sensitivity [24,25].

3.3 Protein identification by ESI nano-HPLC-MS

In order to analyze the samples by bottom-up proteomics we first trypsinized the protein extracts and interpreted the raw data obtained from nano-HPLC-MS/MS using Proteome Discoverer (Supplemental Table S2). Overall, the number of peptide spectrum matches (PSMs) and peptides found in the Trizol samples was similar to the ones detected in the UT samples (PSMs: T1=4339, T2=4432; T3=3389; UT1=4295, UT2=4432, UT3=4327p=0.46, and peptides: T1=3117, T2=3108, T3=2292; UT1=3074, UT2=3156, UT3=3090, p=0.43). From the identified peptides, we inferred a similar number of proteins in the Trizol and the UT samples (proteins identified based on at least one proteospecific peptide: Trizol=814; UT=798; proteins identified based on at least two proteospecific peptides: Trizol=459; UT=507). However, except for sample T3, Trizol extraction revealed a higher number of protein identifications compared to UT. Improved protein identification was also reported by others, recommending the use of Trizol to the mapping of whole proteomes [21,26], with the advantage that Trizol can simultaneously extract RNA, DNA, and protein from the same sample leading to sample economy, which is especially useful when dealing with small and precious samples (biopsies, sorted cells etc.) [8,14].

Overall, there was a 77.49 % overlap between the proteins identified using UT or Trizol (Supplemental Figure 2). For both protein extraction methods, we found an 80% overlap between the proteins identified in the three bioreplicates used which is in the range of the technical variance across the replicates.

To assess the similarity of the protein profiles between the two extraction methods, proteins were classified using Protein Center (Figure 3). Top cellular components covered by the extracted proteins were very similar for the two extraction methods - cytoplasm (T=23.63% /UT=23.88%),

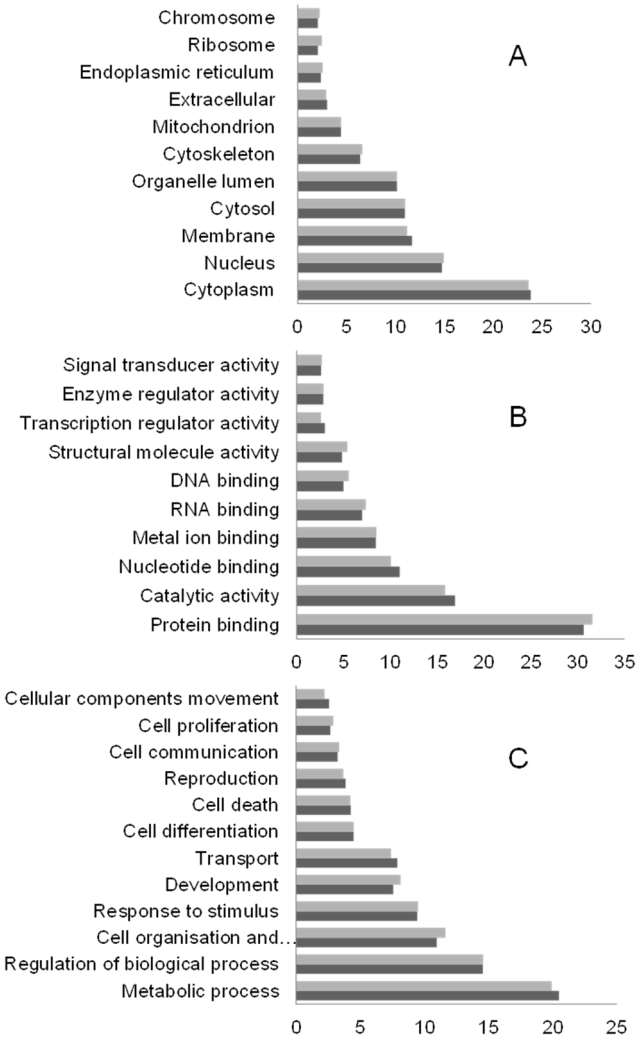

Fig. 4 ProteinCenter assignment of proteins to the corresponding (A) cellular components, (B) molecular functions and (C) biological processes covered by the proteins extracted either by UT (dark bars) or by Trizol (grey bars).

nucleus (14.94% / 14.78%) and membrane (11.19% / 11.73%). Likhite et al. identified the inability to analyze nuclear proteins as a major limitation of the Trizol method [9], but it seems that our modification to the Trizol protocol also improved coverage of the nuclear protein fraction. The presence of membrane proteins in the Trizol samples shows that the modification to the Trizol manufacturer's protocol led to the improvement of the reconstitution of membrane proteins, which sometimes poses difficulty during extraction and dissolution. Top molecular functions of the identified proteins were protein binding (T=31.59%, UT=30.67%), catalytic activity (15.85% / 16.92%) and nucleotide binding (10.04% / 11.01%) and top biological processes covered by the identified proteins were metabolic (19.92% / 20.5%), regulation of biological processes (14.58% / 14.56%) and cell organization and biogenesis (11.64% / 10.97%) and all were very similarly covered by both extraction methods. Molecular functions and biological processes were also similarly covered by the proteins extracted by both methods.

4. Concluding Remarks

In the present study we have performed a Trizol protein extraction from a cell culture and compared it to traditional urea/ thiourea lysis buffer extraction resulting in comparable quality of the proteins fractions recovered by the two methods, with the major advantage that the Trizol protocol also enables simultaneous RNA and DNA extraction from the same sample.

We have demonstrated that sufficient amounts of protein for further analysis can be extracted from S9 cells for further proteomics applications using a modified Trizol protocol. Our study highlights that the modified Trizol extraction allows rapid protein extraction with minimal protein degradation by proteolysis and yields highly pure protein extracts, compatible with many types of protein analysis techniques such as 1D-, 2D-PAGE, Western blot analysis and most importantly LC-tandem mass spectrometry.

5. Supplementary material

Supplementary data and information is available at: http://www.jiomics.com/index.php/jio/rt/suppFiles/185/0

Supplementary Table S1 LC-MS/MS parameters and presentation of proteomic data

Supplementary Table S2 List of all proteins extracted from human airway epithelial cell line S9 using Trizol and urea/ thiourea

Supplementary Fig. S1 Venn diagram depicting the protein overlap for the merged results from 3 biological replicates for UT and Trizol extraction methods.

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