Western blot, ELISA and enzymatic assays of reference proteins for subcellular fractionation

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ABSTRACT

Subcellular fractionation of mammalian cells has been applied for the study of morphology, composition, structure and interactions between organelles, cellular and molecular biology and, more recently, the cell composition through omics approaches


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Advantages of fractionation comprise but are not limited to obtaining fractions enriched in certain compartments for the study of cellular processes in vitro


. .


, locate and track proteins
and analyze post-translational modifications (PTM) of proteins processed along the secretory pathway and protein composition of organelles.

- **Sun FC, Wei S, Li CW, Chang YS, Chao CC, Lai YK (2006).** Localization of GRP78 to mitochondria under the unfolded protein response. *The Biochemical journal.*  
  [http://10.1042/BJ20051916](http://10.1042/BJ20051916)


- **Foster LJ, de Hoog CL, Zhang Y, Zhang Y, Xie X, Mootha VK, Mann M (2006).** A mammalian organelle map by protein correlation profiling.. *Cell.*  
  [http://10.1016/j.cell.2006.03.022](http://10.1016/j.cell.2006.03.022)

  [https://doi.org/10.1155/2012/832569](https://doi.org/10.1155/2012/832569)

Despite the existence of a wide variety of cell fractionation protocols and techniques, most of them are based on the identification or assignment of a certain isolated fraction to one or more subcellular compartments from the enrichment of this fraction in certain markers, the which are mostly protein. The quantification of these protein markers can be done through western blot, ELISA, enzymatic assays or proteomic studies. The present protocol is focused in the identification of proteins markers from several organelles, by WB, ELISA and enzymatic assays. The enrichment of isolated fractions in endoplasmic reticulum (ER), cytosol, nucleus, mitochondria, plasma membrane (PM), cis-Golgi and trans-Golgi can be assessed by detection of 78 kDa glucose-regulated protein (Grp78), glyceraldehyde 3-phosphate dehydrogenase (Gapdh), histone H3, heat shock protein 60 (Hsp60), flotilin 1, golgin A5 and golgin-97, respectively, by WB, and also by ELISA in case of golgin-97. Enrichment of peroxisomes in fractions collected from sucrose gradients can be assessed by a catalase assay adapted from...
This protocol can be applied to any mammalian cell line during subcellular fractionation in order to quantify the enrichment of several organelles in the isolated fractions.


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KEYWORDS
Subcellular fractionation, mammalian cells, endoplasmic reticulum, cytosol, nucleus, mitochondria, plasma membrane, cis-Golgi, trans-Golgi, peroxisomes, Grp78, Gapdh, histone H3, Hsp60, flotilin 1, golgin A5, golgin-97, catalase

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GUIDELINES
Always, wear gloves for this procedure to avoid contamination of WB membranes, and use blunt tweezers for membrane manipulation in order to avoid its damage.

MATERIALS TEXT
MATERIALS

☐ Sodium bicarbonate Sigma

Aldrich Catalog #S6014

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MilliQ water

Contributed by users

Bovine Serum Albumin (BSA) Sigma

Aldrich Catalog #A7906

High-binding 96-well microplates greiner bio-one Catalog #655061

Sodium Chloride Sigma Catalog #S9888

Tween 20 Sigma Catalog #P1379

Potassium chloride Sigma

Aldrich Catalog #P9333

Disodium phosphate Sigma

Aldrich Catalog #S7907

Hydrochloric acid Sigma

Aldrich Catalog #320331-500ML

Sodium carbonate Sigma

Aldrich Catalog #222321

Hydrogen Peroxide Sigma

Aldrich Catalog #H1009-500ML

Thiourea Sigma

Aldrich Catalog #T8656

Acetic acid Sigma

Aldrich Catalog #695092

Triton X-100 Sigma

Aldrich Catalog #X100

Immobilon-P PVDF Membrane, 0.45um, roll Millipore

Sigma Catalog #IPVH00010

SuperSignal™ West Pico PLUS Chemiluminescent Substrate Thermo

Fisher Catalog #34579

DTT Millipore

Sigma Catalog #DTT-RO

SIGMAFAST™ Protease Inhibitor Tablets Sigma

Aldrich Catalog #S8820

Single channel micropipette Transferpette® S adjustable CE-IVD DE-M 20 - 200 µl

BRAND Catalog #705878

Single channel micropipette Transferpette® S adjustable CE-IVD DE-M 100 - 1.000 µl

BRAND Catalog #705880

Potassium phosphate monobasic Sigma

Aldrich Catalog #P0662

Methanol Sigma

Aldrich Catalog #322415

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Tris base Sigma  
Sigma Aldrich Catalog #TRIS-RO

Glycine Sigma  
Sigma Aldrich Catalog #410225

Sodium dodecyl sulfate Sigma  
Sigma Aldrich Catalog #L3771

Ponceau S Sigma  
Sigma Aldrich Catalog #P3504

Skim Milk Powder Serva, Germany Catalog #42590.02

Endoplasmic Reticulum Fraction Western Blot Cocktail Abcam Catalog #ab139415

Golgin 97 antibody [C2C3] C-term Genetex Catalog #GTX114445

Flotillin 1 antibody [C3] C-term Genetex Catalog #GTX104769

HSP60 antibody Genetex Catalog #GTX110089

GOLGA5 antibody [N2C2] Internal Genetex Catalog #GTX104255

Goat Anti-Rabbit IgG H&L (HRP) Abcam Catalog #ab205718

Escherichia coli (Migula) Castellani and Chalmers ATCC Catalog #53606

CHO DP-12 clone#1933 [CHO DP-12 clone#1933 alL8.92 NB 28605/12] ATCC Catalog #CRL-12444

Urea Sigma  
Sigma Aldrich Catalog #U5128

CHAPS Sigma  
Sigma Aldrich Catalog #10810118001

SIGMAFAST™ OPD Sigma  
Sigma Aldrich Catalog #P9187

PYREX Glass Rimless Test Tube 12x75mm The Science Company® Catalog #NC-0993

Potassium phosphate dibasic Sigma  
Sigma Aldrich Catalog #P3786

Corning® microvolume pipet tips Sigma  
Sigma Aldrich Catalog #CLS4894

BRAND® pipette tips bulk Sigma  
Sigma Aldrich Catalog #Z740030

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Membrane preparation and protein transfer for WB assays | 1h 45m

1. Cut the PVDF membrane to the dimensions of the polyacrylamide gel from which the proteins are to be transferred.

2. Soak the PVDF membrane in 100% methanol for 00:05:00, with a constant agitation.

3. Discard methanol and soak the membrane in MilliQ water for 00:02:00, with a constant agitation.

4. Discard water and soak the membrane in transfer buffer (20 Milimolar (mM) Tris, 154 Milimolar (mM) glycine, 0.08% [w/v] SDS, 20% [v/v] methanol) for 00:05:00, with a constant agitation.

5. After removing the polyacrilamide gel from the electrophoresis chamber, rinse it with MilliQ water 3 times to remove excess electrophoresis buffer salts and detergents, and soak it in transfer buffer for 00:05:00, with a constant agitation.

6. Assemble the transfer cassette according to the reference manual of the Trans-Blot SD Semi-Dry Transfer Cell (Bio-Rad, CA, USA).
Proceed to transfer at 20 V for 30-60 min according to the size of the protein to be detected and equipment performance.

Wet the membrane in Ponceau staining (0.5% [w/v] Ponceau S, 1% [v/v] acetic acid), with constant agitation, until detection of protein bands.

Prior to WB assay, it should be corroborated that the protein transfer was successful. This step is optional but highly recommended to ensure that low or no detection of the protein of interest is due to its concentration in the experimental samples and not to poor transfer.

Rinse the membrane with MilliQ water 3 times to eliminate excess Ponceau staining, and destain it with Tris-Glycine pH 8.3 (25 Mm Tris, 192 Mm Glycine, 0.1% [w/v] SDS) until all previous staining has disappeared. Rinse the membrane with MilliQ water 3 times to eliminate excess detergent.

Storage the membrane in phosphate buffer (137 Mm NaCl, 2.7 Mm KCl, 8.1 Mm Na₂HPO₄, 1.8 Mm KH₂PO₄) at 4 °C until use.

Detection of Grp78, Gapdh and histone H3 by WB

Block the membrane in 5% (w/v) skimmed milk, 0.05% (v/v) Tween-20 in phosphate buffer (137 Mm NaCl, 2.7 Mm KCl, 8.1 Mm Na₂HPO₄, 1.8 Mm KH₂PO₄), for 01:00:00 at Room temperature, with constant agitation.
13 Incubate the membrane with Endoplasmic Reticulum Fraction Western Blot Cocktail (Abcam, Cambridge, MA, USA), diluted 2000 times in 0.5% (w/v) BSA, 0.05% (v/v) Tween-20 in phosphate buffer, for 01:00:00 at Room temperature, with constant agitation.

14 Repeat step 12.

15 Incubate the membrane with horseradish peroxidase (HRP) Conjugated Secondary Antibody Cocktail (Abcam, Cambridge, MA, USA), diluted 2500-fold in 0.5% (w/v) BSA, 0.05% (v/v) Tween-20 in phosphate buffer, for 01:00:00 at Room temperature, with constant agitation.

16 Repeat step 12.

17 Wash the membrane in phosphate buffer for 00:05:00, and wet it in a 1:1 (v/v) mix of Luminol/Enhancer Solution and Stable Peroxide Solution from SuperSignal West Pico Chemiluminescent Substrate kit (Thermo Fisher Scientific, Waltham, MA, USA).

18 Acquire the WB image in a LI-COR C-DiGit Chemiluminescence Western Blot Scanner by using Image Studio software in high sensitivity mode (LI-COR Biosciences, Lincoln, NE, USA).

Alternatively, the resulting bands can be visualized by X-ray films or other CCD camera–based digital imaging instruments.
Incubate the membrane with the corresponding primary antibody, diluted in 3% skimmed milk, 0.1% (v/v) Tween-20 in TBS, **Overnight** at **4 °C**, with constant agitation.

Anti golgin-97 (GTX114445, GeneTex, CA, USA), anti flotilin 1 (GTX104769, GeneTex, CA, USA), anti Hsp60 (GTX110089, GeneTex, CA, USA) and anti golgin A5 (GTX104255, GeneTex, CA, USA) antibodies are diluted 2 000, 2 000, 10 000 and 2 000 times, respectively.

Wash the membrane 3 times in 0.1% (v/v) Tween-20 in TBS for **00:05:00** in each wash, with constant agitation. In the cases of anti Hsp60 and anti golgin A5 antibodies, the time of each wash should be extended to **00:10:00**.

Incubate the membrane with an anti-rabbit IgG conjugated to HRP, used as secondary antibody and diluted 2000 times in 3% skimmed milk, 0.1% (v/v) Tween-20 in TBS, for **01:00:00** at **Room temperature**, with constant agitation. Any other anti-rabbit secondary antibody, conjugated to the HRP enzyme, may be used at the manufacturer’s recommended dilution.

Repeat step 21.

Repeat steps 17-18.

Detection of golgin-97 by ELISA

Coat ELISA high binding plates (Greiner Bio-One GmbH, Austria) with **4 µg** of *E. coli* ATCC S3606 and CRL-12444 cell homogenates, and samples from differential centrifugation, all diluted in a final volume of **200 µl** of...
0.050 Molarity (M) sodium carbonate-bicarbonate buffer, pH 9.6, for 16:00:00 at 4 °C.

Homogenates from E. coli and CRL-12444 cells are used as negative and positive controls of the assay, respectively. E. coli ATCC 53606 can be replaced by any other E. coli strain, and CRL-12444 by any other CHO cell line as well.

To obtain E. coli and CHO cell homogenates, centrifuge CHO cells at 185 x g, 4°C, 00:05:00, and E. coli cells at 8161 x g, 4°C, 00:10:00, wash cell pellets twice in phosphate buffer (137 Millimolar (mM) NaCl, 2.7 Millimolar (mM) KCl, 8.1 Millimolar (mM) Na₂HPO₄, 1.8 Millimolar (mM) KH₂PO₄) and lyse them by solubilization in isoelectric focusing buffer (IEF, 7 Molarity (M) urea, 2 Molarity (M) thiourea, 2% [w/v] CHAPS, 40 Millimolar (mM) dithiothreitol) supplemented with 10% (v/v) of SigmaFast Protease Inhibitor Cocktail. Sonicate the lysates twice for 00:01:00 at 10 µm, and centrifuge at 16000 x g, 4°C, 00:25:00. Storage at -20 °C until use.

Wash the plates 5 times with 200 µl per well of 0.05% (v/v) Tween-20 in phosphate buffer (137 Millimolar (mM) NaCl, 2.7 Millimolar (mM) KCl, 8.1 Millimolar (mM) Na₂HPO₄, 1.8 Millimolar (mM) KH₂PO₄) at Room temperature.

Block the plates with 200 µl of 1% (w/v) BSA and 0.05% Tween-20 in phosphate buffer, for 01:00:00 at Room temperature.

Repeat step 26.

Incubate the plates with anti golgin-97 antibody (GTX114445, GeneTex, CA, USA), diluted 2000-fold in 1% (w/v) BSA and 0.05% Tween-20 in phosphate buffer, for 02:00:00 at Room temperature, with 100 µl per well.

Repeat step 26.
31 Incubate the plates with an anti-rabbit IgG conjugated to HRP, used as secondary antibody and diluted 1000 times in 1% (w/v) BSA and 0.05% Tween-20 in phosphate buffer, and incubate for **01:30:00** at **Room temperature** with **100 µl** per well.

32 Repeat step 26.

33 Add **100 µl** per well of SigmaFast o-phenylenediamine dihydrochloride (OPD) substrate (Sigma-Aldrich, Merck KGaA, Darmstadt, Germany), prepared according to manufacturer’s recommendations, and incubate for **00:15:00** at **Room temperature**.

Other HRP substrates used for ELISA can be used as well.

34 Stop the enzymatic reaction by addition of **50 µl** of HCl 10% (v/v) per well.

35 Read the absorbance at 490 nm.

Enzymatic assay of catalase **30m**

36 Mix **20 µl** of each fraction with **30 µl** of **50 Milimolar (mM) phosphate buffer (pH 7.0)**, **29 Milimolar (mM) KH₂PO₄**, **21 Milimolar (mM) K₂HPO₄**, and **50 µl** of 1% (v/v) Triton X-100, and place in a 12x75 mm glass test tube.

This assay has been optimized to measure catalase activity in fractions collected from sucrose gradients. The assay was adapted from


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Add 50 µl of 30% (v/v) hydrogen peroxide to each tube and mix.

After 00:05:00 incubation, measure the height of foam column.

Calculate the specific activity of catalase (mm/mg) as the ratio between foam height (mm) and the protein quantity added to the tube (mg).