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*

Amodio G, Renna M, Paladino S, Venturi C, Tacchetti C, Moltedo O, Franceschelli S, Mallardo M, Bonatti S, Remondelli P (2009). Endoplasmic reticulum stress reduces the export from the ER and alters the architecture of post-ER compartments.. The international journal of biochemistry & cell biology.
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and protein composition of organelles

Gilchrist A, Au CE, Hiding J, Bell AW, Fernandez-Rodriguez J, Lesimple S, Nagaya H, Roy L, Gosline SJC, Hallett M, Paiement J, Kearney RE, Nilsson T, Bergeron JJM (2006). Quantitative Proteomics Analysis of the Secretory Pathway. Cell.
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. 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

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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

Iwase T, Tajima A, Sugimoto S, Okuda K, Hironaka I, Kamata Y, Takada K, Mizunoe Y (2013). A simple assay for measuring catalase activity: a visual approach.. Scientific reports. https://doi.org/10.1038/srep03081

. 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.

EXTERNAL LINK

https://doi.org/10.1371/journal.pone.0237930

THIS PROTOCOL ACCOMPANIES THE FOLLOWING PUBLICATION

Pérez-Rodriguez S, Ramírez-Lira MdJ, Wulff T, Voldbor BG, Ramírez OT, Trujillo-Roldán MA, Valdez-Cruz NA (2020) Enrichment of microsomes from Chinese hamster ovary cells by subcellular fractionation for its use in proteomic analysis. PLoS ONE 15(8): e0237930. doi: 10.1371/journal.pone.0237930

DOI

dx.doi.org/10.17504/protocols.io.bgc4jsyw

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MANUSCRIPT CITATION please remember to cite the following publication along with this protocol

Pérez-Rodriguez S, Ramírez-Lira MdJ, Wulff T, Voldbor BG, Ramírez OT, Trujillo-Roldán MA, Valdez-Cruz NA (2020) Enrichment of microsomes from Chinese hamster ovary cells by subcellular fractionation for its use in proteomic analysis. PLoS ONE 15(8): e0237930. doi: 10.1371/journal.pone.0237930

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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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36988

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

NAME	CATALOG #	VENDOR
Sodium bicarbonate	S6014	Sigma Aldrich
MilliQ water		
Bovine Serum Albumin (BSA)	A7906	Sigma Aldrich
High-binding 96-well microplates	655061	greiner bio-one
Sodium Chloride	S9888	Sigma
Tween 20	P1379	Sigma
Potassium chloride	P9333	Sigma Aldrich
Disodium phosphate	S7907	Sigma Aldrich
Hydrochloric acid	320331-500ML	Sigma – Aldrich
Sodium carbonate	222321	Sigma – Aldrich
Hydrogen Peroxide	H1009-500ML	Sigma Aldrich
Thiourea	T8656	Sigma Aldrich
Acetic acid	695092	Sigma Aldrich
Triton X-100	X100	Sigma Aldrich
Immobilon-P PVDF Membrane, 0.45um, roll	IPVH00010	Millipore Sigma
SuperSignal™ West Pico PLUS Chemiluminescent Substrate	34579	Thermo Fisher
DTT	DTT-RO	Millipore Sigma
SIGMAFAST™ Protease Inhibitor Tablets	S8820	Sigma Aldrich
Single channel micropipette Transferpette® S adjustable CE-IVD DE-M 20 - 200 µl	705878	BRAND
Single channel micropipette Transferpette® S adjustable CE-IVD DE-M 100 - 1.000 µl	705880	BRAND
Potassium phosphate monobasic	P0662	Sigma Aldrich
Methanol	322415	Sigma Aldrich
Tris base	TRIS-RO	Sigma Aldrich
Glycine	410225	Sigma Aldrich
Sodium dodecyl sulfate	L3771	Sigma Aldrich
Ponceau S	P3504	Sigma Aldrich
Skim Milk Powder	42590.02	Serva, Germany
Endoplasmic Reticulum Fraction Western Blot Cocktail	ab139415	Abcam
Golgin 97 antibody [C2C3] C-term	GTX114445	Genetex
Flotillin 1 antibody [C3] C-term	GTX104769	Genetex
HSP60 antibody	GTX110089	Genetex
GOLGA5 antibody [N2C2] Internal	GTX104255	Genetex
Goat Anti-Rabbit IgG H&L (HRP)	ab205718	Abcam
Escherichia coli (Migula) Castellani and Chalmers	53606	ATCC

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NAME	CATALOG #	VENDOR
CHO DP-12 clone#1933 [CHO DP-12 clone#1933 aIL8.92 NB 28605/12]	CRL-12444	ATCC
Urea	U5128	Sigma Aldrich
CHAPS	10810118001	Sigma Aldrich
SIGMAFAST™ OPD	P9187	Sigma Aldrich
PYREX Glass Rimless Test Tube 12x75mm	NC-0993	The Science Company®
Potassium phosphate dibasic	P3786	Sigma Aldrich
Corning® microvolume pipet tips	CLS4894	Sigma Aldrich
BRAND® pipette tips bulk	Z740030	Sigma Aldrich
BrandTech™ BRAND™ Pipet Tips 50 to 1000uL	13-889-145	Fisher Scientific
Single channel micropipette Transferpette® S adjustable CE-IVD DE-M 05 - 10 µl	705870	BRAND
Multi-channel micropipette Transferpette® S-8 adjustable CE-IVD DE-M 20 - 200 µl	705910	BRAND
Dressing tissue forceps	F4267	Sigma Aldrich

EQUIPMENT

NAME	CATALOG #	VENDOR
Trans-Blot SD Semi-Dry Transfer Cell	1703940	Bio-rad Laboratories

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. 1m

2 5m

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

3 2m

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

4 5m

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

5 7m

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.

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6 Assemble the transfer cassette according to the reference manual of the Trans-Blot SD Semi-Dry Transfer Cell (Bio-Rad, CA, USA). 5m

Trans-Blot SD Semi-Dry Transfer Cell
Protein transfer
Bio-Rad 1703940

PDF

7 Proceed to transfer at 20 V for 30-60 min according to the size of the protein to be detected and equipment performance. 1h

8 10m

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.

9 10m

Rinse the membrane with MilliQ water 3 times to eliminate excess Ponceau staining, and destain it with Tris-Glycine pH 8.3 ([M]**25 Milimolar (mM)** Tris, [M]**192 Milimolar (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.

10 Storage the membrane in phosphate buffer ([M]**137 Milimolar (mM)** NaCl, [M]**2.7 Milimolar (mM)** KCl, [M]**8.1 Milimolar (mM)** Na_2HPO_4, [M]**1.8 Milimolar (mM)** KH_2PO_4) at **4 °C** until use.

Detection of Grp78, Gapdh and histone H3 by WB 4h 42m

11 1h

Block the membrane in 5% (w/v) skimmed milk, 0.05% (v/v) Tween-20 in phosphate buffer ([M]**137 Milimolar (mM)** NaCl, [M]**2.7 Milimolar (mM)** KCl, [M]**8.1 Milimolar (mM)** Na_2HPO_4, [M]**1.8 Milimolar (mM)** KH_2PO_4), for **01:00:00** at **Room temperature** , with constant agitation.

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12 30m

Wash the membrane 3 times in 0.05% (v/v) Tween-20 in phosphate buffer, for **00:10:00** in each wash, with constant agitation.

13 1h

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 30m

Repeat step 12.

15 1h

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 30m

Repeat step 12.

17 6m

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 6m

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.

Detection of golgin-97, golgin A5, flotilin 1 and HSP60 by WB 18h 42m

19 1h

Block the membrane in 3% skimmed milk, 0.1% (v/v) Tween-20 in Tris-buffered saline (TBS, [M]**137 Milimolar (mM)** NaCl, [M]**20 Milimolar (mM)** Tris-HCl, pH**7.6**) for **01:00:00** at **Room temperature** , with constant agitation.

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20 16h

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.

21 15m

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** .

22 1h

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.

23 15m

Repeat step 21.

24 12m

Repeat steps 17-18.

Detection of golgin-97 by ELISA 21h 10m

25 16h

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Coat ELISA high binding plates (Greiner Bio-One GmbH, Austria) with **4 µg** of *E. coli* ATCC 53606 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 Milimolar (mM)** NaCl, **2.7 Milimolar (mM)** KCl, **8.1 Milimolar (mM)** Na_2HPO_4, **1.8 Milimolar (mM)** KH_2PO_4) and lyse them by solubilization in isoelectric focusing buffer (IEF, **7 Molarity (M)** urea, **2 Molarity (M)** thiourea, 2% [w/v] CHAPS, **40 Milimolar (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.

26 5m

Wash the plates 5 times with **200 µl** per well of 0.05% (v/v) Tween-20 in phosphate buffer (**137 Milimolar (mM)** NaCl, **2.7 Milimolar (mM)** KCl, **8.1 Milimolar (mM)** Na_2HPO_4, **1.8 Milimolar (mM)** KH_2PO_4) at **Room temperature**.

27 1h

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**.

28 5m

Repeat step 26.

29 2h

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.

5m

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30

Repeat step 26.

31 1h 30m

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 5m

Repeat step 26.

33 20m

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. 5m

35 Read the absorbance at 490 nm. 2m

Enzymatic assay of catalase 30m

36 5m

Mix **20 µl** of each fraction with **30 µl** of **[M]50 Milimolar (mM)** phosphate buffer (**[M]21 Milimolar (mM)** KH_2PO_4, **[M]29 Milimolar (mM)** K_2HPO_4), **pH7.0** , 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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37 5m

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

38 10m

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

39 10m

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

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