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

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Saumel Perez Rodriguez¹, María De Jesús Ramírez-Lira¹, Tune Wulff², Bjørn Gunnar Voldbor², Octavio T Ramírez³, Mauricio A Trujillo-Roldán¹, Norma A Valdez-Cruz¹

¹Departamento de Biología Molecular y Biotecnología, Instituto de Investigaciones Biomédicas, Universidad Nacional Autónoma de México;

²The Novo Nordisk Foundation Center for Biosustainability, Technical University of Denmark;

³Departamento de Medicina Molecular y Bioprocesos, Instituto de Biotecnología, Universidad Nacional Autónoma de México

Saumel Perez Rodriguez

Departamento de Biología Molecular y Biotecnología, Institut...

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

CITATION

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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[10.1016/j.cell.2006.10.036](https://doi.org/10.1016/j.cell.2006.10.036)

CITATION

Jadot M, Boonen M, Thirion J, Wang N, Xing J, Zhao C, Tannous A, Qian M, Zheng H, Everett JK, Moore DF, Sleat DE, Lobel P (2017). Accounting for Protein Subcellular Localization: A Compartmental Map of the Rat Liver Proteome.. Molecular & cellular proteomics : MCP.

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<https://doi.org/10.1074/mcp.M116.064527>

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

CITATION

Itzhak DN, Tyanova S, Cox J, Borner GH (2016). Global, quantitative and dynamic mapping of protein subcellular localization.. eLife.

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Christoforou A, Mulvey CM, Breckels LM, Geladaki A, Hurrell T, Hayward PC, Naake T, Gatto L, Viner R, Martinez Arias A, Lilley KS (2016). A draft map of the mouse pluripotent stem cell spatial proteome.. Nature communications.

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<https://doi.org/10.1038/ncomms9992>

CITATION

Mulvey CM, Breckels LM, Geladaki A, Britovšek NK, Nightingale DJH, Christoforou A, Elzek M, Deery MJ, Gatto L, Lilley KS (2017). Using hyperLOPIT to perform high-resolution mapping of the spatial proteome.. Nature protocols.

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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*

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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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Balch WE, Dunphy WG, Braell WA, Rothman JE (1984). Reconstitution of the transport of protein between successive compartments of the Golgi measured by the coupled incorporation of N-acetylglucosamine. *Cell*.

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Dominguez M, Fazel A, Dahan S, Lovell J, Herno L, Claude A, Melançon P, Bergeron JJ (1999). Fusogenic domains of golgi membranes are sequestered into specialized regions of the stack that can be released by mechanical fragmentation.. *The Journal of cell biology*.

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[10.1083/jcb.145.4.673](https://doi.org/10.1083/jcb.145.4.673)

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, locate and track proteins

CITATION

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*.

LINK

[10.1042/BJ20051916](https://doi.org/10.1042/BJ20051916)

and analyze post-translational modifications (PTM) of proteins processed along the secretory pathway

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Erra MC, Iodice L, Lotti LV, Bonatti S (1999). Cell fractionation analysis of human CD8 glycoprotein transport between endoplasmic reticulum, intermediate compartment and

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LINK

[10.1006/cbir.1999.0420](https://doi.org/10.1006/cbir.1999.0420)

and protein composition of organelles

CITATION

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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[10.1016/j.cell.2006.10.036](https://doi.org/10.1016/j.cell.2006.10.036)

CITATION

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.

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[10.1016/j.cell.2006.03.022](https://doi.org/10.1016/j.cell.2006.03.022)

CITATION

Peng F, Zhan X, Li MY, Fang F, Li G, Li C, Zhang PF, Chen Z (2012). Proteomic and bioinformatics analyses of mouse liver microsomes.. International journal of proteomics.

LINK

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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 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

CITATION

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.

LINK

<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.

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

MATERIALS

- ☒ Sodium bicarbonate **Sigma Aldrich Catalog #S6014**
- ☒ 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 - 1.00 µl BRAND Catalog #705880
✕	Potassium phosphate monobasic Sigma Aldrich Catalog #P0662
✕	Methanol Sigma Aldrich Catalog #322415
✕	Tris base Sigma Aldrich Catalog #TRIS-RO
✕	Glycine Sigma Aldrich Catalog #410225
✕	Sodium dodecyl sulfate Sigma Aldrich Catalog #L3771
✕	Ponceau S 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 aIL8.92 NB 28605/12] ATCC Catalog #CRL-12444
✕	Urea Sigma Aldrich Catalog #U5128
✕	CHAPS Sigma Aldrich Catalog #10810118001
✕	SIGMAFAST™ OPD Sigma Aldrich Catalog #P9187
✕	PYREX Glass Rimless Test Tube 12x75mm The Science Company® Catalog #NC-0993
✕	Potassium phosphate dibasic Sigma Aldrich Catalog #P3786
✕	Corning® microvolume pipet tips Sigma Aldrich Catalog #CLS4894
✕	BRAND® pipette tips bulk Sigma Aldrich Catalog #Z740030
✕	BrandTech™ BRAND™ Pipet Tips 50 to 1000uL Fisher Scientific Catalog #13-889-145
✕	Single channel micropipette Transferpette® S adjustable CE-IVD DE-M 05 - 10 µl BRAND Catalog #705870

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 Soak the PVDF membrane in 100% methanol for 00:05:00, with a constant agitation. 5m

- 3 Discard methanol and soak the membrane in MilliQ water for 00:02:00, with a constant agitation. 2m

- 4 Discard water and soak the membrane in transfer buffer (20 millimolar (mM) Tris, 154 millimolar (mM) glycine, 0.08% [w/v] SDS, 20% [v/v] methanol) for 00:05:00, with a constant agitation. 5m

- 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. 7m

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

Equipment

Trans-Blot SD Semi-Dry Transfer Cell

NAME

Protein transfer

TYPE

Bio-Rad

BRAND

1703940

SKU

<https://www.bio-rad.com/es-mx/product/trans-blot-sd-semi-dry-transfer-cell?ID=b92801aa-76b7-45c9-825e-672589d01b00>

LINK

- 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 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. 10m

*

Note

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 Rinse the membrane with MilliQ water 3 times to eliminate excess Ponceau staining, and destain it with Tris-Glycine pH 8.3 ([M] 25 millimolar (mM) Tris, [M] 192 millimolar (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. 10m

*

- 10 Storage the membrane in phosphate buffer ([M] 137 millimolar (mM) NaCl, [M] 2.7 millimolar (mM) KCl, [M] 8.1 millimolar (mM) Na₂HPO₄, [M] 1.8 millimolar (mM) KH₂PO₄) at 4 °C until use.

Detection of Grp78, Gapdh and histone H3 by WB

4h 42m

- 11 Block the membrane in 5% (w/v) skimmed milk, 0.05% (v/v) Tween-20 in phosphate buffer ([M] 137 millimolar (mM) NaCl, [M] 2.7 millimolar (mM) KCl, [M] 8.1 millimolar (mM) Na₂HPO₄, [M] 1.8 millimolar (mM) KH₂PO₄), for 01:00:00 at Room temperature, with constant agitation.

1h

- 12 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.

30m

- 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.

1h

- 14 Repeat step 12.

30m

- 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.

1h

- 16 Repeat step 12.

30m

- 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).

6m

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).

6m

Note

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 w..

18h 42m

19

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

1h

20

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.

16h

Note

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

Wash the membrane 3 times in 0.1% (v/v) Tween-20 in TBS for 00:05:00 in each wash, with constant agitation.

15m

Note

In the cases of anti Hsp60 and anti golgin A5 antibodies, the time of each wash should be extended to 00:10:00 .

22

Incubate the membrane with an anti-rabbit IgG conjugated to HRP, used as secondary antibody

1h

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.

Note

Any other anti-rabbit secondary antibody, conjugated to the HRP enzyme, may be used at the manufacturer's recommended dilution.

23 Repeat step 21.

15m

24 Repeat steps 17-18.

12m

Detection of golgin-97 by ELISA

21h 10m

25 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.

16h

Note

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.

Note

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.

- 26** 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. 5m
-
- 27** 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. 1h
-
- 28** Repeat step 26. 5m
-
- 29** 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. 2h
-
- 30** Repeat step 26. 5m
-
- 31** Incubate the plates with an anti-rabbit IgG conjugated to HRP, used as secondary antibody and 1h 30m

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 μ L** per well.

32

Repeat step 26.

5m

33

Add **100 μ L** 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**.

20m

Note

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

34

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

5m

35

Read the absorbance at 490 nm.

2m

Enzymatic assay of catalase

30m

36

Mix **20 μ L** of each fraction with **30 μ L** of **50 millimolar (mM)** phosphate buffer (**21 millimolar (mM)** KH_2PO_4 , **29 millimolar (mM)** K_2HPO_4 , **pH 7.0**), and **50 μ L** of 1% (v/v) Triton X-100, and place in a 12x75 mm glass test tube.

5m

Note

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

CITATION

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.

LINK

<https://doi.org/10.1038/srep03081>

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

5m

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

10m

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

10m