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Subcellular fractionation of suspension Chinese Hamster Ovary (CHO) cells

PLOS One

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ABSTRACT

Subcellular fractionation is a widely adopted technique to study cellular biology, and localization and functions of several proteins

CITATION

Baudhuin P, Berthet J. Electron microscopic examination of subcellular fractions. II. Quantitative analysis of the mitochondrial population isolated from rat liver. J. Cell Biol..

LINK

[10.1083/jcb.35.3.631](https://doi.org/10.1083/jcb.35.3.631)

CITATION

Butler WH, Judah JD (1970). Preparation of isolated rat liver mitochondria for electron microscopy. J Cell Biol..

LINK

[10.1083/jcb.44.2.278](https://doi.org/10.1083/jcb.44.2.278)

PROTOCOL integer ID:
36882

Keywords: Chinese hamster ovary CHO cells, subcellular fractionation, sucrose gradient, proteomics

CITATION

Vance JE (1990). Phospholipid synthesis in a membrane fraction associated with mitochondria. J Biol Chem..

LINK

[2332429](#)

CITATION

Spatuzza C, Renna M, Faraonio R, Cardinali G, Martire G, Bonatti S, Remondelli P (2004). Heat shock induces preferential translation of ERGIC-53 and affects its recycling pathway. The Journal of biological chemistry.

LINK

[10.1074/jbc.M401860200](#)

, and, more recently, it has been applied previous to proteomics to quantify low concentration proteins, and characterize many subcellular 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 REE, Nilsson T, Bergeron JJM (2006). Quantitative Proteomics Analysis of the Secretory Pathway. Cell.

LINK

[10.1016/j.cell.2006.10.036](#)

CITATION

Yates JR 3rd, Gilchrist A, Howell KE, Bergeron JJM (2005). Proteomics of organelles and large cellular structures. Nat Rev Mol Cell Biol..

LINK

[10.1038/nrm1711](#)

CITATION

Stasyk T, Huber LA (2004). Zooming in: fractionation strategies in proteomics.. Proteomics.

LINK

[10.1002/pmic.200401048](https://doi.org/10.1002/pmic.200401048)

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

<https://doi.org/10.1155/2012/832569>

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.

LINK

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

CITATION

Andreyev AY, Shen Z, Guan Z, Ryan A, Fahy E, Subramaniam S, Raetz CR, Briggs S, Dennis EA (2010). Application of proteomic marker ensembles to subcellular organelle identification. Molecular & cellular proteomics : MCP.

LINK

<https://doi.org/10.1074/mcp.M900432-MCP200>

. Since CHO cells play a central role for recombinant protein (RP) production, a deeper study of their cellular biology is mandatory in order to construct new cell sub-lines with a more robust RP expression phenotype. Therefore, the goal of the present protocol is to provide the necessary basis for separation of subcellular organelles from suspension Chinese hamster ovary (CHO) cells for a subsequent proteomic study of isolated fractions. Differential and isopycnic centrifugation based sections are presented for enrichment of different subcellular compartments, together with a precipitation section for recovering of proteins from isolated

fractions. Isopycnic centrifugation is based on discontinuous sucrose gradients, of which one is adapted from the literature for the separation of nuclear and mitochondrial precipitates

CITATION

Graham J (2004). Isolation of Mitochondria from a Homogenate using a Sucrose Density Gradient. eLS.

LINK

[10.1038/npg.els.0003807](https://doi.org/10.1038/npg.els.0003807)

, and the other is designed in our laboratory for the separation of the microsomal precipitate. This protocol provides a novel discontinuous sucrose gradient for the separation of the components of the microsomes, which increases the separation efficiency with respect to previously reported protocols

CITATION

Erra MC, Iodice L, Lotti LV, Bonatti S (1999). Cell fractionation analysis of human CD8 glycoprotein transport between endoplasmic reticulum, intermediate compartment and Golgi complex in tissue cultured cells.. Cell biology international.

LINK

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

GUIDELINES

This subcellular protocol is divided in 4 sections: cell disruption, differential centrifugation, isopycnic centrifugation and protein precipitation. Although it has been developed for enrichment of subcellular compartments in discontinuous sucrose gradients, pellets from differential centrifugation can be used as well, without a subsequent separation in density gradients. The enrichment of organelles by differential and isopycnic centrifugation is summarized in the following image:

Enrichment of subcellular compartments from CHO cell homogenates by differential and isopycnic centrifugation based protocol.

MATERIALS

MATERIALS

⊗ Sodium Chloride Sigma Catalog #S9888

⊗ Potassium chloride Sigma Aldrich Catalog #P9333

✕	Disodium phosphate Sigma Aldrich Catalog #S7907
✕	Acetone Sigma Aldrich Catalog #179124
✕	Falcon™ 15mL Conical Centrifuge Tubes Fisher Scientific Catalog #14-959-53A
✕	Ethylenediaminetetraacetic acid Sigma Aldrich Catalog #E9884
✕	Sucrose Sigma Aldrich Catalog #S7903
✕	PMSF (Phenylmethylsulfonyl fluoride) Sigma Aldrich Catalog #10837091001
✕	Falcon 50mL Conical Centrifuge Tubes Fisher Scientific Catalog #14-432-22
✕	Trypan Blue Sigma Aldrich Catalog #T8154
✕	SIGMAFAST™ Protease Inhibitor Tablets Sigma Aldrich Catalog #S8820
✕	PIPETTE TIPS 10 - 100 µL YELLOW SUITABLE FOR EPPENDORF 96 PIECES PER RACK greiner bio-one Catalog #685280
✕	PIPETTE TIP 100-1000 µL BLUE BULK PACKED 250 PCS./BAG greiner bio-one Catalog #686290
✕	BRAND® microcentrifuge tube 1.5 mL with lid PP Sigma Aldrich Catalog #Z336769
✕	Neubauer chamber Electron Microscopy Sciences Catalog #68052-14
✕	Cover Glass 22 x 22mm No. 1 1 oz Thomas Scientific Catalog #1169W09
✕	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
✕	Homogeniser 30 ml CarlRoth Catalog #TT61.1
✕	Accessories Pestle for homogeniser Suitable for: Volume 30 ml Carl Roth Catalog #TT67.1
✕	Potassium phosphate monobasic Sigma Aldrich Catalog #P0662
✕	HEPES Sigma Aldrich Catalog #H7523

Cell disruption

1h 51m

- 1 Quantify cell concentration and viability during cell culture to reach 300-700 millions of viable cells, with a viability higher than 90%. 10m

- 2 Centrifuge cells at 185 x g, 4°C, 00:05:00, in 50 mL conical tubes. 5m

- 3 Discard supernatant gently to avoid any cell lost. 1m

- 4 Resuspend cellular pellet to about $3-7 \times 10^7$ cells/ml in 10 mL of cold phosphate buffer (137 millimolar (mM) NaCl, 2.7 millimolar (mM) KCl, 8.1 millimolar (mM) Na₂HPO₄, 1.8 millimolar (mM) KH₂PO₄). 25m

Note

Calculate total viable cells present in the pellet by multiplying collected culture volume and viable cell concentration. Pool as many cell pellets as necessary to complete the total number of viable cells required for the procedure.

- 5 Centrifuge cells at 185 x g, 4°C, 00:05:00, in 50 mL conical tubes. 5m

- 6 Discard supernatant gently. 1m

7 Resuspend cell pellet in 10 mL of cold phosphate buffer.

5m

8 Repeat steps 5 and 6 once.

6m

9 Resuspend cell pellet in 8 mL of hypotonic buffer (10 millimolar (mM) HEPES, 1 millimolar (mM) EDTA, 7.4).

5m

10 Incubate the cell suspension during 00:30:00 On ice , inverting tube every 00:10:00 .

30m

11 Add PMSF to a final concentration of 1 millimolar (mM) and 500 μ L of prepared Protease Inhibitor Tablets to every 350 millions of cells.

1m

Note

To prepare Protease Inhibitor Tablets, dissolve one tablet in 100 mL of sterile MilliQ water, filter through a 0.2 μ m filter and distribute 1 mL aliquots in 1-2 ml eppendorf tubes. Keep at -20 °C until use.

12 Transfer the cells to a Dounce homogenizer, and disrupt them by 25 strokes, On ice .

15m

Note

Since cell disruption must be optimized for each cell line in order to reach maximum efficiency and organelle integrity, number of strokes should be carefully evaluated in a case by case basis.

Note

Another cell disruption method, such as nitrogen cavitation

CITATION

Storrie B, Amadden E (1990). Isolation of subcellular organelles. Guide to Protein Purification.

LINK

[https://doi.org/10.1016/0076-6879\(90\)82018-W](https://doi.org/10.1016/0076-6879(90)82018-W)

CITATION

Gottlieb RA, Adachi S (2000). Nitrogen cavitation for cell disruption to obtain mitochondria from cultured cells. Methods in enzymology.

LINK

[10.1016/s0076-6879\(00\)22022-3](https://doi.org/10.1016/s0076-6879(00)22022-3)

CITATION

Simpson RJ (2010). Disruption of cultured cells by nitrogen cavitation. Cold Spring Harbor protocols.

LINK

<https://doi.org/10.1101/pdb.prot5513>

, sonication

CITATION

Bahnemann J, Kayo S, Wahrheit J, Heinzle E, Pörtner R, Zeng AP (2013). In search of an effective cell disruption method to isolate intact mitochondria from Chinese hamster ovary cells. Eng. Life Sci..

LINK

[10.1002/elsc.201200182](https://doi.org/10.1002/elsc.201200182)

and Balch homogenizer

CITATION

Balch WE, Rothman JE (1985). Characterization of protein transport between successive compartments of the Golgi apparatus: Asymmetric properties of donor and acceptor activities in a cell-free system. Arch. Biochem. Biophys..

LINK

[10.1016/0003-9861\(85\)90046-3](https://doi.org/10.1016/0003-9861(85)90046-3)

, can be evaluated, depending on availability.

13 Restore isotonicity immediately by adding 2 mL of 45% (w/v) sucrose solution, prepared in the hypotonic buffer, to the cell homogenate.

1m

14 Transfer disrupted cells back again to 50 mL tubes and homogenize this suspension by inversion.

1m

Differential centrifugation

1d 1h 36m

15 Distribute the cell homogenate in 1 mL aliquots into 1.5 mL microcentrifuge tubes.

3m

16 Centrifuge at 3000 x g, 4°C, 00:10:00.

10m

17 Collect supernatant and distribute it to new 1.5 mL microcentrifuge tubes. Tag the pellet as Nuclear Fraction and maintain it On ice . 3m

18 Centrifuge the collected supernatant at 9000 x g, 4°C, 00:10:00 . 10m

19 Collect and pool the new supernatant in 13.2 mL , Open-Top Thinwall Ultra-Clear/Polypropylene Tube, 14 x 89 mm. Tag the pellet as Mitochondrial Fraction and maintain it On ice . 5m

20 Centrifuge the pooled supernatant at 100000 x g, 4°C, 01:00:00 , in a SW40Ti rotor, using an Optima XE-90 Ultracentrifuge. 1h

Note

Any other ultracentrifuge compatible with this rotor and running settings can be used.

Safety information

Read all product manuals and consult with Beckman Coulter-trained personnel before attempting to operate the instrument. Do not attempt to perform any procedure before carefully reading all instructions. Always follow product labeling and manufacturer's recommendations. If in doubt as to how to proceed in any situation, contact your Beckman Coulter Representative.

21 Collect the supernatant in 15 mL conical tubes. Tag the pellet as Microsomal Fraction, and 5m

supernatant as Cytosol, and maintain both On ice .

22

Precipitate proteins from supernatant (cytosol) immediately, as detailed below, and storage protein pellet at -80 °C until use.

1d

Note

If the proteomic analysis will make use of the differential fractionation samples, without any further separations, storage nuclear, mitochondrial and microsomal pellets at -80 °C until use. In case that these pellets will be separated in density gradients, do not freeze these samples, and proceed to separation immediately after their obtaining.

Isopycnic centrifugation

1d 8h 30m

23

Prepare discontinuous sucrose gradients On ice by depositing the indicated volumes on the tube wall, drop by drop, slowly.

3h

Note

Use a gradient of 1 mL of 60% (w/v) and 3 mL of each solution of 55, 40 and 30% (w/v) sucrose, placed from the bottom to the top of the tube, for separation of nuclear and mitochondrial pellets

CITATION

Graham JM (2004). Isolation of Mitochondria from a Homogenate using a Sucrose Density Gradient. eLS.

LINK

[10.1038/npg.els.0003807](https://doi.org/10.1038/npg.els.0003807)

Discontinuous sucrose gradient (30-60%), adapted from literature, for separation of subcellular organelles from nuclear and mitochondrial pellets.

A second one formed by 1 mL 60% (w/v) and 2.5 mL of each solution of 45, 35, 30 and 10% (w/v) sucrose, placed from the bottom to the top of the tube, will be used for separation of microsomal pellet.

A novel discontinuous sucrose gradient (10-60%) for separation of subcellular organelles from microsomal pellet.

Sucrose solutions should always be deposited in decreasing order of concentration. The interface between these solutions should always be visible, except in the case of 30/35% where it is difficult to distinguish. Discard gradients if interfaces are not clearly visible. Use

13.2 mL, Open-Top Thinwall Ultra-Clear/Polypropylene Tube, 14 x 89 mm to prepare gradients. All solutions should be kept on ice throughout the procedure, and prepared according to the percentage of sucrose indicated in each case (w/v), in a buffer with a final composition of 10 millimolar (mM) HEPES and 1 millimolar (mM) EDTA, 7.4

24 Pool all the mitochondrial pellets by resuspending them gently in 1 mL of isotonic buffer [0.25 Molarity (M) sucrose (w/v), 10 millimolar (mM) HEPES, 1 millimolar (mM) EDTA, 7.4].

5m

25 Divide nuclear pellets in 4 groups, and pool each group by resuspending them gently in 1 mL of isotonic buffer.

20m

26 Resuspend the microsomal pellet in 0.5 mL of isotonic buffer.

5m

27 Add each resuspended pellet to the top of the corresponding sucrose gradient and centrifuge at 217874 x g, 4°C, 03:00:00 (max), in a SW40Ti rotor, using an Optima XE-90 Ultracentrifuge, with maximal and medium acceleration and deceleration rates, respectively.

3h

Note

Any other ultracentrifuge compatible with this rotor and running settings can be used.

28 Collect visible bands in each gradient by using a Pasteur pipette, 1 mL micropipette tips, a peristaltic pump, or by tube puncture or any other method suitable for fraction collection.

2h

Expected result

Three bands should be seen on each gradient, except for the nuclear gradient, where 4 bands are resolved.

Note

The densest band on the nuclear gradient, located at the bottom of the tube, should be discarded as it represents cell debris.

29

Precipitate proteins from each fraction collected from sucrose gradients by the protocol detailed below, and storage protein pellets at -80 °C until use.

1d

Protein precipitation

30

Add 260 µL of 4 Molarity (M) NaCl to 10 mL of sample in 50 mL conical tubes, and homogenize by vortex.

Note

This section has been optimized to precipitate complex protein mixtures from CHO cells

CITATION

Crowell AM, Wall MJ, Doucette AA (2013). Maximizing recovery of water-soluble proteins through acetone precipitation.. Analytica chimica acta.

LINK

<https://doi.org/10.1016/j.aca.2013.08.005>

. The obtained pellet can be shipped to a Proteomic Facility, at Room temperature , for its processing and analysis. Total volumes should be changed according to available sample, always maintaining the indicated proportion of solutions.

31

Add 40 mL of cold 80% (v/v) acetone and homogenize by vortex or inversions. Acetone should be kept at -20 °C, at least 16:00:00 before precipitation.

Note

Acetone percentage can be increased to 100% (v/v) if protein recovery is not enough for proteomic analysis.

Safety information

Use gloves, safety glasses and protective clothing. Do not eat, drink or smoke during use. Keep away from heat, hot surfaces, open flames, sparks. Keep container tightly closed.

32

Incubate at -20 °C during 16:00:00.

Note

Incubation for a longer time could be necessary if protein recovery is not enough for proteomic analysis.

33

Centrifuge at 8228 x g, 4°C, 00:30:00. Discard supernatant.

34

Add 40 mL of 80% (v/v) acetone and homogenize by vortex or inversions.

35

Centrifuge at 8228 x g, 4°C, 00:30:00. Discard supernatant.

36

Repeat steps 34 and 35.

37

Centrifuge at 8228 x g, 4°C, 00:01:00 and discard all supernatant carefully by using a micropipette.

38

Dry the pellet at Room temperature during 00:05:00 .

Note

Do not over-dry the precipitate as this decreases the solubilization of the proteins in a later step.

39

Tag and seal the tube to be shipped to Proteomic Facility.