Aug 26, 2020

Subcellular fractionation of suspension Chinese Hamster Ovary (CHO) cells

PLOS One

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1 Works for me dx.doi.org/10.17504/protocols.io.bf9sjr6e

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Departamento de Biología Molecular y Biotecnología, Institut...

ABSTRACT

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

Baudhuin P, Berthet J. Electron microscopic examination of subcellular fractions. II. Quantitative analysis of the mitochondrial population isolated from rat liver. J. Cell Biol.. http://10.1083/jcb.35.3.631

Butler WH, Judah JD (1970). Preparation of isolated rat liver mitochondria for electron microscopy. J Cell Biol.. http://10.1083/jcb.44.2.278

Vance JE (1990). Phospholipid synthesis in a membrane fraction associated with mitochondria. J Biol Chem.. http://2332429

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. http://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: Saumel Perez Rodriguez, MarÃÂa De JesÃÂºs RamÃÂrez-Lira, Tune Wulff, BjÃÂ¸rn Gunnar Voldbor, Octavio T RamÃÂrez, Mauricio A Trujillo-RoldÃÂ¡n, Norma A Valdez-Cruz (08/26/2020). Subcellular fractionation of suspension Chinese Hamster Ovary (CHO) cells . https://dx.doi.org/10.17504/protocols.io.bf9sjr6e

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. http://10.1016/j.cell.2006.10.036

Yates JR 3rd, Gilchrist A, Howell KE, Bergeron JJM (2005). Proteomics of organelles and large cellular structures. Nat Rev Mol Cell Biol.. http://10.1038/nrm1711

Stasyk T, Huber LA (2004). Zooming in: fractionation strategies in proteomics.. Proteomics. http://10.1002/pmic.200401048

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

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

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. 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,

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together with a precipitation section for recovering of proteins from isolated fractions. Isopycnic centrifugation is based on discontinuos sucrose gradients, of which one is adapted from the literature for the separation of nuclear and mitochondrial precipitates

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

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. http://10.1006/cbir.1999.0420

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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.bf9sjr6e

PROTOCOL CITATION

Saumel Perez Rodriguez, María De Jesús Ramírez-Lira, Tune Wulff, Bjørn Gunnar Voldbor, Octavio T Ramírez, Mauricio A Trujillo-Roldán, Norma A Valdez-Cruz 2020. Subcellular fractionation of suspension Chinese Hamster Ovary (CHO) cells . **protocols.io** https://dx.doi.org/10.17504/protocols.io.bf9sjr6e

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

KEYWORDS

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

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CREATED

May 11, 2020

LAST MODIFIED

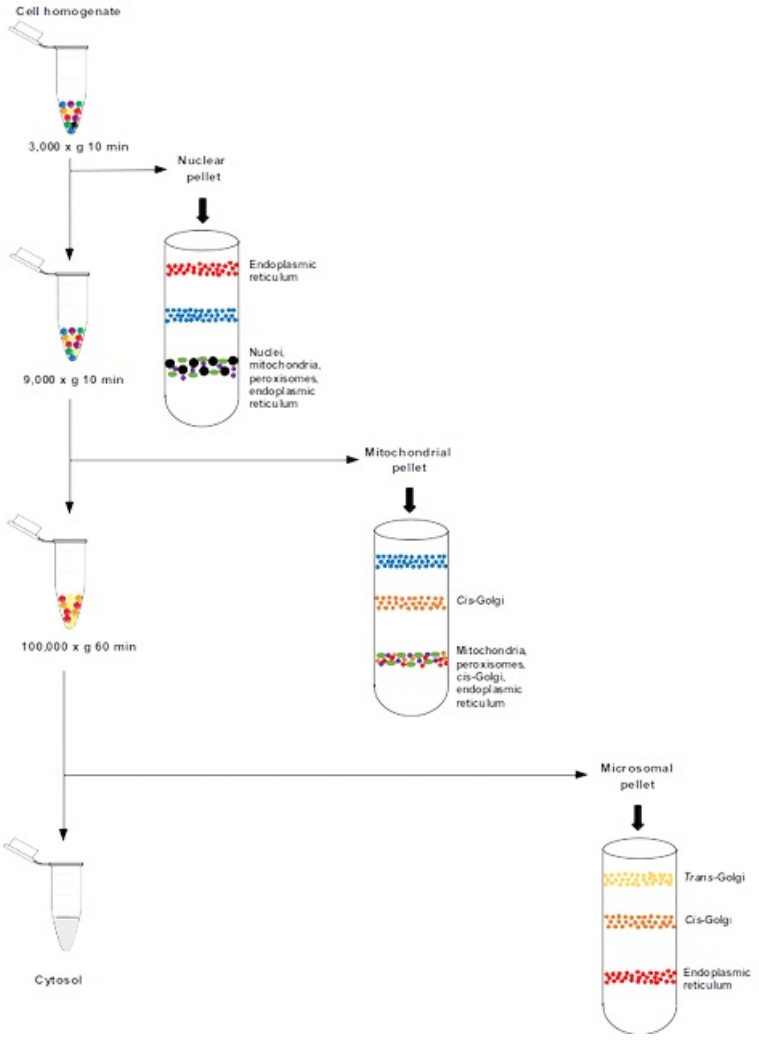
Aug 26, 2020

PROTOCOL INTEGER ID

36882

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 discontinuos 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

Citation: Saumel Perez Rodriguez, MarÃÂa De JesÃÂºs RamÃÂrez-Lira, Tune Wulff, BjÃÂ¸rn Gunnar Voldbor, Octavio T RamÃÂrez, Mauricio A Trujillo-RoldÃÂ¡n, Norma A Valdez-Cruz (08/26/2020). Subcellular fractionation of suspension Chinese Hamster Ovary (CHO) cells . https://dx.doi.org/10.17504/protocols.io.bf9sjr6e

MATERIALS

NAME	CATALOG #	VENDOR
Sodium Chloride	S9888	Sigma
Potassium chloride	P9333	Sigma Aldrich
Disodium phosphate	S7907	Sigma Aldrich
Acetone	179124	Sigma Aldrich
Falcon™ 15mL Conical Centrifuge Tubes	14-959-53A	Fisher Scientific
Ethylenediaminetetraacetic acid	E9884	Sigma Aldrich
Sucrose	S7903	Sigma Aldrich
PMSF (Phenylmethylsulfonyl fluoride)	10837091001	Sigma Aldrich
Falcon 50mL Conical Centrifuge Tubes	14-432-22	Fisher Scientific
Trypan Blue	T8154	Sigma Aldrich
SIGMAFAST™ Protease Inhibitor Tablets	S8820	Sigma Aldrich
PIPETTE TIPS 10 - 100 µL YELLOW SUITABLE FOR EPPENDORF 96 PIECES PER RACK	685280	greiner bio-one
PIPETTE TIP 100-1000 µL BLUE BULK PACKED 250 PCS./BAG	686290	greiner bio-one
BRAND® microcentrifuge tube 1.5 mL with lid PP	Z336769	Sigma Aldrich
Neubauer chamber	68052-14	Electron Microscopy Sciences
Cover Glass 22 x 22mm No. 1 1 oz	1169W09	Thomas Scientific
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
Homogeniser 30 ml	TT61.1	CarlRoth
Accessories Pestle for homogeniser Suitable for: Volume 30 ml	TT67.1	Carl Roth
Potassium phosphate monobasic	P0662	Sigma Aldrich
HEPES	H7523	Sigma Aldrich
SW 40 Ti Swinging-Bucket Rotor Package	331301	Beckman Coulter
13.2 mL Open-Top Thinwall Ultra-Clear Tube 14 x 89mm	344059	Beckman Coulter

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 5m

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

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

4 25m

Resuspend cellular pellet to about 3-7 x 10^7 cells/ml in **10 mL** of cold 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).

Citation: Saumel Perez Rodriguez, MarÃÂa De JesÃÂºs RamÃÂrez-Lira, Tune Wulff, BjÃÅ¸rn Gunnar Voldbor, Octavio T RamÃÂrez, Mauricio A Trujillo-RoldÃÂ¡n, Norma A Valdez-Cruz (08/26/2020). Subcellular fractionation of suspension Chinese Hamster Ovary (CHO) cells . https://dx.doi.org/10.17504/protocols.io.bf9sjr6e

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 

5m

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

6 Discard supernatant gently.

1m

7 

5m

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

8 Repeat steps 5 and 6 once.

6m

9 

5m

Resuspend cell pellet in **8 mL** of hypotonic buffer (**10 Milimolar (mM)** HEPES, **1 Milimolar (mM)** EDTA, **pH 7.4**).

10 

30m

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

11 Add PMSF to a final concentration of **1 Milimolar (mM)** and **500 µl** of prepared Protease Inhibitor Tablets to every 350 millions of cells.

1m

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

12 

15m

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

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.

Another cell disruption method, such as nitrogen cavitation

Storrie B, Amadden E (1990). Isolation of subcellular organelles. Guide to Protein Purification. https://doi.org/10.1016/0076-6879(90)82018-W

Gottlieb RA, Adachi S (2000). Nitrogen cavitation for cell disruption to obtain mitochondria from cultured cells. Methods in enzymology. http://10.1016/s0076-6879(00)22022-3

Simpson RJ (2010). Disruption of cultured cells by nitrogen cavitation. Cold Spring Harbor protocols. https://doi.org/10.1101/pdb.prot5513

, sonication

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.. http://10.1002/elsc.201200182

and Balch homogenizer

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.. http://10.1016/0003-9861(85)90046-3

, can be evaluated, depending on availability.

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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  3m

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

16  10m

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

17  3m

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

18  10m

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

19  5m

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** .

20  1h

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

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

Citation: Saumel Perez Rodriguez, MarÃÂa De JesÃÂºs RamÃÂrez-Lira, Tune Wulff, BjÃÅ¸rn Gunnar Voldbor, Octavio T RamÃÂrez, Mauricio A Trujillo-RoldÃÂ¡n, Norma A Valdez-Cruz (08/26/2020). Subcellular fractionation of suspension Chinese Hamster Ovary (CHO) cells . https://dx.doi.org/10.17504/protocols.io.bf9sjr6e

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 

5m

Collect the supernatant in **15 mL** conical tubes. Tag the pellet as Microsomal Fraction, and supernatant as Cytosol, and maintain both **On ice** .

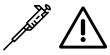
22 

1d

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

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 

3h

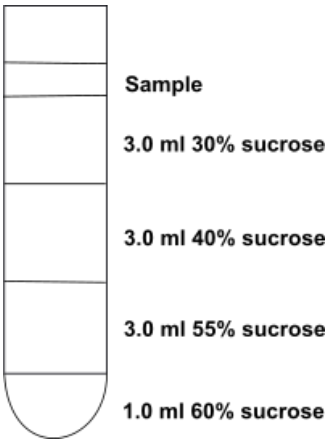
Prepare discontinuous sucrose gradients **On ice** by depositing the indicated volumes on the tube wall, drop by drop, slowly.

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

Graham JM (2004). Isolation of Mitochondria from a Homogenate using a Sucrose Density Gradient. eLS. http://10.1038/npg.els.0003807

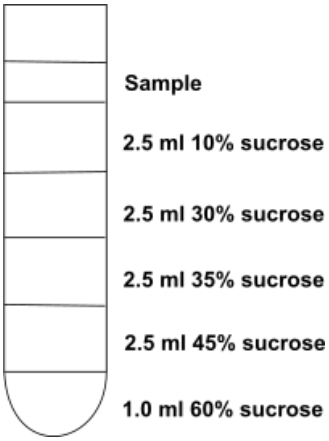
.

Citation: Saumel Perez Rodriguez, MarÃÂa De JesÃÂºs RamÃÂrez-Lira, Tune Wulff, BjÃÅ¸rn Gunnar Voldbor, Octavio T RamÃÂrez, Mauricio A Trujillo-RoldÃÂ¡n, Norma A Valdez-Cruz (08/26/2020). Subcellular fractionation of suspension Chinese Hamster Ovary (CHO) cells . https://dx.doi.org/10.17504/protocols.io.bf9sjr6e



Discontinuos 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 discontinuos 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 [M]**10 Milimolar (mM)** HEPES and [M]**1 Milimolar (mM)** EDTA, pH**7.4** .

24 5m

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

20m

Citation: Saumel Perez Rodriguez, MarÃÂa De JesÃÂºs RamÃÂrez-Lira, Tune Wulff, BjÃÅ¸rn Gunnar Voldbor, Octavio T RamÃÂrez, Mauricio A Trujillo-RoldÃÂ¡n, Norma A Valdez-Cruz (08/26/2020). Subcellular fractionation of suspension Chinese Hamster Ovary (CHO) cells . https://dx.doi.org/10.17504/protocols.io.bf9sjr6e

25

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

26 5m

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

27 3h

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.

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

28 2h

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.

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

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

29 1d

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

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.

Citation: Saumel Perez Rodriguez, MarÃÂa De JesÃÂºs RamÃÂrez-Lira, Tune Wulff, BjÃÅ¸rn Gunnar Voldbor, Octavio T RamÃÂrez, Mauricio A Trujillo-RoldÃÂ¡n, Norma A Valdez-Cruz (08/26/2020). Subcellular fractionation of suspension Chinese Hamster Ovary (CHO) cells . https://dx.doi.org/10.17504/protocols.io.bf9sjr6e

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

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

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

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** .

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.

Citation: Saumel Perez Rodriguez, MarÃÂa De JesÃÂºs RamÃÂrez-Lira, Tune Wulff, BjÃÅ¸rn Gunnar Voldbor, Octavio T RamÃÂrez, Mauricio A Trujillo-RoldÃÂ¡n, Norma A Valdez-Cruz (08/26/2020). Subcellular fractionation of suspension Chinese Hamster Ovary (CHO) cells . https://dx.doi.org/10.17504/protocols.io.bf9sjr6e

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** .

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.

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