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Complete Cell Fractionation in U937 Cells in the Absence of High Speed Centrifugation

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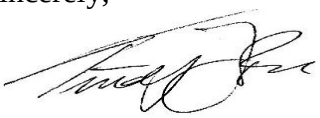
Dear Ms. Troyer,

Thank you indeed for the invitation to publish our methods in *JoVE*. We are flattered by your invitation and agree that JoVE is the perfect forum through which to share our unique methods with others in the scientific community. In this manuscript we describe our method for subcellular fractionation of eukaryotic cells. Our procedure is distinct from subcellular fractionation methods based on detergent solubilization and high speed centrifugation (the two main methods currently employed for subcellular fractionation). Detergent- and high speed centrifugation-based methods have several drawbacks including the inability for detergent-based fractionation to distinguish between cellular organelles and plasma membrane and the prohibitive costs of high speed centrifuges. Our procedure does not include these drawbacks as it is based on digitonin extraction of the cytoplasm followed by differential centrifugation in a standard, refrigerated, benchtop centrifuge. Therefore, we believe this procedure will be useful to investigators who wish to perform an efficient yet cost-effective subcellular fractionation.

Due to the potentially wide-reaching implications of this procedure for others in the scientific community, we believe that *JoVE* is an ideal journal for its publication. Thank you again for the invitation to publish as well as your time and consideration.

Sincerely,



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TITLE:

Cell Fractionation of U937 Cells in the Absence of High-speed Centrifugation

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KEYWORDS:

Cellular fractionation, Mitochondria, Membrane, Cytoplasm, Organelles, Centrifugation, U937

SUMMARY:

Here, we present a protocol to isolate the plasma membrane, cytoplasm and mitochondria of U937 cells without the use of high-speed centrifugation. This technique can be used to purify subcellular fractions for subsequent examination of protein localization *via* immunoblotting.

ABSTRACT:

In this protocol we detail a method to obtain subcellular fractions of U937 cells without the use of ultracentrifugation or indiscriminate detergents. This method utilizes hypotonic buffers, digitonin, mechanical lysis and differential centrifugation to isolate the cytoplasm, mitochondria and plasma membrane. The process can be scaled to accommodate the needs of researchers, is inexpensive and straightforward. This method will allow researchers to determine protein localization in cells without specialized centrifuges and without the use of commercial kits, both of which can be prohibitively expensive. We have successfully used this method to separate cytosolic, plasma membrane and mitochondrial proteins in the human monocyte cell line U937.

INTRODUCTION:

Reliable identification of protein localization is often necessary when examining molecular pathways in eukaryotic cells. Methods to obtain subcellular fractions are utilized by researchers to more closely examine cellular components of interest.

The majority of existing cell fractionation methods generally fall into two broad categories, detergent-based[1,2] and ultracentrifugation-based[3–5], which can be differentiated by speed, precision and cost. Detergent based protocols rely on the use of buffers with increasing detergent

strength to solubilize distinct components of the cell. This is a rapid and convenient method for processing samples and can be cost effective if the number and size of samples are small. Detergent-based kits can be purchased to isolate cytoplasmic, membrane/organelle (mixed fraction), and nuclear fractions from cells. However, several drawbacks associated with these kits limit their usefulness to researchers. They are designed to easily isolate one or two components of the cell, but are incapable of isolating all fractions from a sample concurrently. The use of detergents means that the plasma membrane and membrane-enclosed organelles will be equally solubilized and, therefore, unable to be separated from one another. An additional complication arises from the proprietary components in these kits which prevents researchers from altering conditions for specific applications. Lastly, they are limited in number of uses and may be prohibitively expensive for larger scale experiments. Non-detergent based kits exist for the isolation of mitochondria, however, they are not designed to isolate plasma membrane and the sample yield is significantly less than that from density centrifugation based isolation protocols[6,7].

Methods that utilize ultracentrifugation to obtain fractions are more time consuming, but often result in purer fractions than detergent-based kits. To isolate plasma membranes from cells without first solubilizing them (resulting in contamination with membrane organelles) requires them to be lysed by a non-detergent method followed by separation of cellular components *via* differential centrifugation—with plasma membrane isolation requiring speeds of 100,000 × *g* to accomplish. In many cases, differential centrifugation must be followed by isopycnic density gradient centrifugation for further separation of cellular fractions or removal of contaminants. While these methods are thorough and modifiable, drawbacks include cost, time consumption, and the need for an ultracentrifuge for separation of fractions and further purification *via* density gradient centrifugation. Most high-speed centrifuges are at a cost that is prohibitive for individual investigators and are often shared, core equipment at academic institutions. Thus, ultracentrifuge availability becomes prohibitive in these situations.

In this fractionation protocol we demonstrate the isolation of subcellular fractions without the use of solubilizing detergents and without high speed centrifugation. This method will allow researchers to isolate the plasma membrane, mitochondria and cytoplasmic components of a eukaryotic cell with minimal contamination between fractions.

PROTOCOL:

1. **Prepare Buffers and Reagents**

NOTE: See **Table 1**.

1.1. Prepare solutions of buffer A, lysis buffer B, sample buffer and digitonin.

1.1.1. Prepare buffer A by adding 8.77 g of NaCl and 50 mL of HEPES (1 M, pH 7.4) to 900 mL deionized water, adjust final volume to 1 L with deionized water.

NOTE: Final concentrations are 150 mM NaCl and 50 mM HEPES.

1.1.2. Prepare lysis buffer B by adding 20 mL of HEPES (1 M, pH 7.4), 0.75 g of KCl, 0.19 g of $MgCl_2$, 2 mL of Ethylenediaminetetraacetic acid (0.5 M EDTA), 2 mL of ethylene glycol-bis(β-aminoethyl ether)-N,N,N',N'-tetraacetic acid (0.5 M EGTA), 38.26 g of mannitol and 23.96 g of sucrose to 900 mL of deionized water, adjust final volume to 1 L with deionized water.

NOTE: Final concentrations are 20 mM HEPES, 10 mM KCl, 2 mM $MgCl_2$, 1 mM EDTA, 1 mM EGTA, 210 mM mannitol and 70 mM sucrose.

1.1.3. Prepare sample buffer by adding 0.01 g of sodium dodecyl sulfate (SDS) to 10 mL of tris-buffered saline (TBS) for a final concentration of 0.1% SDS.

1.1.4. Prepare a stock solution of digitonin by adding 25 mg of digitonin to 100 mL of deionized water (final concentration is 250 µg/mL).

1.1.5. Store all buffer solutions at 4 °C and digitonin at -20 °C until the start of experiment.

1.2. Prepare fresh solutions of protease and phosphatase inhibitors to be added to buffer solutions prior to addition to cells.

1.2.1. Prepare a stock solution of phenylmethanesulfonyl fluoride (PMSF) by adding 17.4 mg of PMSF to 1 mL of 100% ethanol (final concentration is 100 mM).

CAUTION: Wear appropriate protective equipment and exercise caution when handling PMSF. PMSF is hazardous if ingested and slightly hazardous in case of skin contact (irritant), eye contact (irritant) or inhalation; it is corrosive to eyes and skin.

1.2.2. Prepare a commercially available protease inhibitor cocktail (100×) according to the manufacturer's instructions (see the **Table of Materials**).

1.2.3. Prepare a stock solution of sodium orthovanadate (SOV) by adding 92 mg of SOV to 1 mL of deionized water (final concentration is 500 mM).

CAUTION: Wear appropriate protective equipment and use caution when handling. SOV is hazardous in case of eye contact (irritant), ingestion or inhalation. Severe over-exposure can result in death.

2. PBS Wash

2.1. Concentrate and wash cells in phosphate-buffered saline (PBS) prior to fractionation.

2.1.1. Centrifuge cell suspension at an appropriate speed to create a pellet. For example, centrifuge a suspension of U937 cells at 400 × g for 10 min.

2.1.2. Remove the supernatant, resuspend cell pellet in room-temperature PBS at a final concentration of 4×10^6 cells/mL and pipette gently to break up clumps.

2.1.3. Centrifuge the cell suspension at 400 × g for 10 min to pellet cells.

2.1.4. Remove the supernatant and resuspend cell pellet in **ice cold** buffer A at a final concentration of 2×10^7 cells/mL.

NOTE: All subsequent steps should be carried out at **4 °C** or on ice and all buffers should be **pre-chilled**.

3. **Cytosolic Protein Isolation**

3.1. Extract cytosolic proteins by incubation with the detergent digitonin.

3.1.1. Immediately prior to resuspension of cells (step 3.1.3) add 10 µL of stock PMSF (100 mM), 10 µL of protease Inhibitor (100×), 2 µL of stock SOV (500 mM) and 100 µL of stock digitonin (250 µg/mL) to 878 µL of buffer A (final concentrations are 1 mM PMSF, 1× Protease Inhibitor, 1 mM SOV and 25 µg/mL digitonin; adjust the final volume as per the number of cells being used). Keep the solution on **ice** until addition to cell pellet.

3.1.2. Centrifuge the cell suspension at 400 × g for 10 min and remove the supernatant.

3.1.3. Resuspend the cell pellet in buffer A solution containing inhibitors and digitonin (prepared in step 3.1.1) at a final concentration of 2×10^7 cells/mL, pipette gently to break up clumps.

3.1.4. Incubate the cell suspension on an end-over-end rotator at 4 °C for 20 min.

3.1.5. Centrifuge the cell suspension at 400 × g for 10 min. Collect the supernatant and place it in a clean centrifuge tube.

3.1.6. Centrifuge the collected supernatant at 18,000 × g for 20 min to pellet cellular debris.

3.1.7. Transfer the supernatant to a clean centrifuge tube.

3.1.8. Repeat steps 3.1.5 and 3.1.6 until no pellet is obtained following centrifugation.

3.1.9. Collect the supernatant containing the **cytosolic proteins** and store it at 4 °C (short term) or -20 °C (long term).

3.2. Remove excess digitonin and cytosolic proteins by centrifugation.

3.2.1. Resuspend the digitonin-permeabilized cell pellet (from step 3.1.5) in buffer A at a final concentration of 4×10^6 cells/mL and pipette gently to break up clumps.

3.2.2. Centrifuge the digitonin-permeabilized cell suspension at 400 × g for 10 min and remove the supernatant.

NOTE: Repeated washes in buffer A can be performed to remove excess cytosolic contaminants.

4. **Cell Homogenization**

4.1. Incubate the cells on ice in lysis buffer B and lyse them by mechanical means.

4.1.1. Immediately prior to resuspension of cells (step 4.1.2) add 10 µL of stock PMSF (100 mM) and 2 µL of stock SOV (500 mM) to 988 µL of lysis buffer B (final concentrations are 1 mM PMSF and 1 mM SOV; adjust final volume to accommodate number of cells being lysed) and keep solution on **ice** until addition to cell pellet.

4.1.2. Resuspend the cell pellet (from step 3.2.2) in **ice cold** lysis buffer B solution containing PMSF and SOV (prepared in step 4.1.1) at a final concentration of 4×10^6 cells/mL.

4.1.3. Incubate the cell suspension on ice for 30 min.

4.1.4. Transfer the cell suspension to a pre-chilled Dounce homogenizer (with a tight-fitting B pestle) on ice and perform 40 passes with the homogenizer pestle using slow, even strokes.

NOTE: Alternatively utilize other means of mechanical cell lysis as detailed in discussion section.

4.1.5. Collect the homogenate and transfer it to a clean centrifuge tube.

4.1.6. Wash the homogenizer pestle and tube with a small volume (1 to 2 mL) of lysis buffer B and add it to the homogenate.

4.1.7. Centrifuge the homogenate at 400 × g (or the minimum speed required to pellet unbroken cells) for 10 min.

4.1.8. Transfer the supernatant to a clean centrifuge tube.

Note: If a significant pellet remains repeat steps 4.1.4 through 4.1.6 to increase the yield of fractions as detailed in the discussion section. The protocol can be paused here, and the homogenate stored at 4 °C for short term (24 h).

5. **Differential Centrifugation**

5.1. Centrifuge the homogenate at increasing speeds to remove cellular debris, isolate mitochondria and membrane fractions.

5.1.1. Centrifuge the supernatant (from step 4.1.8) at 500 × g for 10 min. Transfer supernatant to a clean centrifuge tube, and discard any pellet.

5.1.2. Centrifuge supernatant (from step 5.1.1) at 1,000 × g for 10 min. Transfer the supernatant to a clean centrifuge tube, and discard any pellet.

5.1.3. Centrifuge the supernatant (from step 5.1.2) at 2,000 × g for 10 min. Transfer the supernatant to a clean centrifuge tube, and discard any pellet.

5.1.4. Centrifuge the supernatant (from step 5.1.3) at 4,000 × g for 15 min. Transfer supernatant to a clean centrifuge tube, **keep** pellet containing mitochondria.

5.1.5. Resuspend the mitochondria pellet in a small volume (0.5–1 mL) of lysis buffer B.

5.1.6. Centrifuge the suspended pellet at 4,000 × g for 15 min. Remove the supernatant and resuspend the **mitochondrial** pellet in the desired final volume of sample buffer (*e.g.*, 250 to 500 µL, depending on the size of the pellet and desired concentration).

5.1.7. Centrifuge the supernatant (from step 5.1.4) at 4,000 × g for 15 min. Transfer the supernatant to a clean centrifuge tube. Repeat this step until no pellet is obtained following centrifugation.

5.1.8. Spin the supernatant at 18,000 × g for 3 h.

5.1.9. Remove the supernatant, and keep the pellet containing membrane proteins. Resuspend the membrane pellet in a small volume (0.5–1 mL) of lysis buffer B.

5.1.10. Centrifuge the suspended pellet at 18,000 × g for 1 h.

5.1.11. Remove the supernatant and resuspend the **membrane** pellet in the desired final volume of sample buffer (250 to 500 µL, depending on the size of the pellet and desired concentration).

5.2. Sonicate the sample pellets for 3 s in an ice bath at a power setting of 5 (50% of 125 W maximum power at 20 kHz, see **the Table of Materials**).

5.3. Store the samples at 4 °C (short term) or -20 °C (long term).

5.4. Examine the samples for purity of fractionation by performing a western blot utilizing antibodies against protein markers found in the cytoplasm, mitochondria and membrane compartments of the cell (refer to the representative results section).

REPRESENTATIVE RESULTS:

Successful fractionation of undifferentiated U937[8] cells grown in suspension was accomplished using the protocol detailed above and illustrated in **Figure 1**. The samples obtained with this

method were subjected to western blotting[9] utilizing a wet transfer method to a polyvinylidene fluoride (PVDF) membrane. The membrane was subsequently probed with antibodies against cytoplasmic, mitochondrial and membrane localized protein markers (**Figure 2**, **Figure 3**, **Figure 4**). The successful extraction of cytoplasmic proteins can be verified by probing the blot with antibodies against the cytosolic protein glyceraldehyde-3-phosphate dehydrogenase[10] (GAPDH), normally localized to the cytoplasm of the cell. As shown by the immunoblot (**Figure 2**; bottom panel, lanes 1 and 2), GAPDH is found only in the digitonin extracted samples and no contamination is observed in the 4,000 × g pellets (**Figure 4**; lanes 3 and 4 on the bottom panel) or 18,000 × g pellets (**Figure 4**; lanes 5 and 6 on the bottom panel). Probing for the voltage-dependent anion channel (VDAC), a protein localized to the outer mitochondrial membrane[11], shows the successful isolation of mitochondria in the 4,000 × g pellets (**Figure 4**; lanes 3 and 4 on the middle panel), while the absence of this protein in other fractions shows the lack of mitochondrial contamination in the 18,000 × g pellets or digitonin-extracted samples. Probing for the Na/K-ATPase α1 subunit, part of an integral membrane heterodimer found primarily in the plasma membrane[12], shows the majority of this protein located in the 18,000 × g pellets (**Figure 4**; lanes 5 and 6 on the top panel). This protein is also detected in the 4,000 × g pellets (**Figure 4**; lanes 3 and 4 of the top panel), suggesting possible contamination with plasma membrane, though this possibility is unlikely at the low speed with which this pellet was obtained. A more plausible explanation is the presence of endoplasmic reticulum (ER) in the 4,000 × g pellets sample, as transport of Na,K-ATPase subunits from the ER to the plasma membrane has been demonstrated by researchers[13]. The lack of Na,K-ATPase α1 protein in the digitonin extracted samples (**Figure 4**; lanes 1 and 2 on the top panel) demonstrates the purity of this fraction.

In contrast to the outcome observed during a successful fractionation (**Figure 2**), improper execution of the protocol can result in cross contamination of cellular components (**Figure 3**, **Figure 4**). A high concentration of Na,K-ATPase α1 protein in the 4,000 × g pellets, when compared to the 18,000 × g pellets (**Figure 3** {top panel, lane 2-3] and **Figure 4B** [top panel, lanes 5-12]), indicates that the organelle fraction has been contaminated with plasma membrane proteins. The presence of GAPDH in any fraction other than the digitonin-extracted cytoplasmic sample (**Figure 3** [bottom panel, lane 4] and **Figure 4A** [bottom panel, lanes 5-12] is an indicator of failure to remove cytoplasmic proteins in subsequent steps.

FIGURE AND TABLE LEGENDS:

Figure 1: Diagram of the Cell Fractionation Protocol. An overview of the cell fractionation protocol represented as a flow chart.

Figure 2: Successful Isolation of U937 Cell Cytoplasm, Organelle, and Membrane Fractions. Western blots of cell fractions isolated from two U937 cell cultures and probed for markers of membrane (Na, K-ATPase α1, top panel), mitochondria (VDAC, middle panel) and cytoplasm (GAPDH, bottom panel).

Figure 3: Failure to Fractionate U937 Cell Components. Western blots of cell fractions isolated from a single U937 cell culture showing contamination of the organelle fraction (lane 2) with Na,

K-ATPase α1 (top panel) and loss of cytoplasmic components into the supernatant of the membrane pellet (lane 4).

Figure 4: Cross Contamination of Cytoplasmic and Membrane Components During Fractionation. (**A**) Fractionation attempt with cytoplasmic cross contamination of GAPDH in organelle (bottom panel, lanes 5-8) and membrane fractions (bottom panel, lanes 9-12). (**B**) Fractionation attempt with plasma membrane contamination of Na, K-ATPase in mitochondrial fractions (top panel, lanes 5-8).

Table 1: Buffers and Solutions. Composition of buffers and required solutions for the procedure.

Table 2: Critical Steps. Summary table of protocol steps, potential issues and possible solutions for troubleshooting the protocol.

DISCUSSION:

The development of this protocol arose from an inability to separate mitochondrial and membrane samples, using commercially available kits, for analysis of protein localization during necroptosis[14]. The primary limitations of premade kits are their inability to be adapted to the needs of individual researchers, their cost per sample and limited number of samples able to be processed. The method presented here can be performed without the use of expensive reagents and without the necessity for expensive equipment. This method can be scaled to accommodate any number of cells and is capable of being altered to suit the needs of researchers. This allows the yield of each fraction to be increased, tailoring of steps to the research being performed and flexibility in execution of the method. The addition of sodium orthovanadate in the buffers can be omitted if researchers are not examining phosphorylation state of proteins in the final samples. The inclusion of SDS in the final buffer can likewise be omitted if researchers wish to further purify the samples *via* density gradient centrifugation. While we have only used this method to successfully fractionate U937 cells, a non-adherent monocyte cell line, the procedure should work with multiple cell lines and tissues, with minor alterations. Cells grown in suspension can be pelleted similarly to the U937 cells detailed here, while adherent cell lines require dissociation from the growth surface prior to beginning this protocol. Similarly, tissues must be thoroughly homogenized prior to fractionation of containing cells. This can be accomplished by utilizing a loose fitting Dounce homogenizer, a Potter-Elvehjem glass-polytetrafluoroethene homogenizer or by other (commercial) methods[15].

There are a number of critical steps in the protocol that need careful attention to obtain optimal results and pure fractions (**Table 2**). The concentration of cells recommended here (steps 2.1.2, 2.1.4, 3.1.3, 3.2.1 and 4.1.2) are specific to U937 cells and were determined through trial and error. These values may need to be adjusted to accommodate different types of cells if suboptimal results are obtained when executing the protocol. During the cytoplasmic extraction step (step 3.1), the concentration of digitonin must be sufficient to permeabilize the plasma membrane without completely lysing the cells. Following this incubation, cells must be thoroughly washed to remove cytosolic proteins from subsequent steps or cross contamination will occur in downstream samples (**Figure 4A**). The homogenization step (step 4.1.4) can be

accomplished with any form of mechanical lysis, although results presented here were obtained with a Dounce homogenizer. An alternative to using a Dounce homogenizer is to repeatedly pass cells through a narrow gauge needle (27 G recommended, 20-40 passes) until sufficient cell lysis is achieved. It may be necessary to increase the number of strokes (or syringe passes) if a large pellet of unbroken cells is obtained after homogenization (step 4.1.7). Researchers will likely need to determine the optimal amount of homogenization for the type of cell being fractionated. Removal of cellular debris following the homogenization must be thorough to avoid contamination of the organelle fraction with plasma membrane components (**Figure 4B**). If this occurs, additional low speed centrifugation steps should be performed to remove cellular debris. During development of this method up to 18 h of centrifugation at 18,000 × g was tested, with minimal increase of plasma membrane yield over the 3 h spins (**Figure 2**, lanes 5-6). Researchers may find it necessary to increase centrifugation times to obtain better yields of the plasma membrane sample.

The method presented here is limited in comparison to the more thorough purification methods involving ultracentrifugation. Without the use of isopycnic density centrifugation it is not possible to separate individual organelles obtained after cell homogenization. While the 4,000 × g fractions contain mitochondria (as evidenced by the presence of VDAC, **Figure 2**), they likely also contain ER, golgi and additional intracellular organelles. The organelle fraction should be verified by the use of additional antibodies to protein markers of other organelles if this is of importance to the research being performed.

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DISCLOSURES:

The authors declare no conflict of interest

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Figure 1

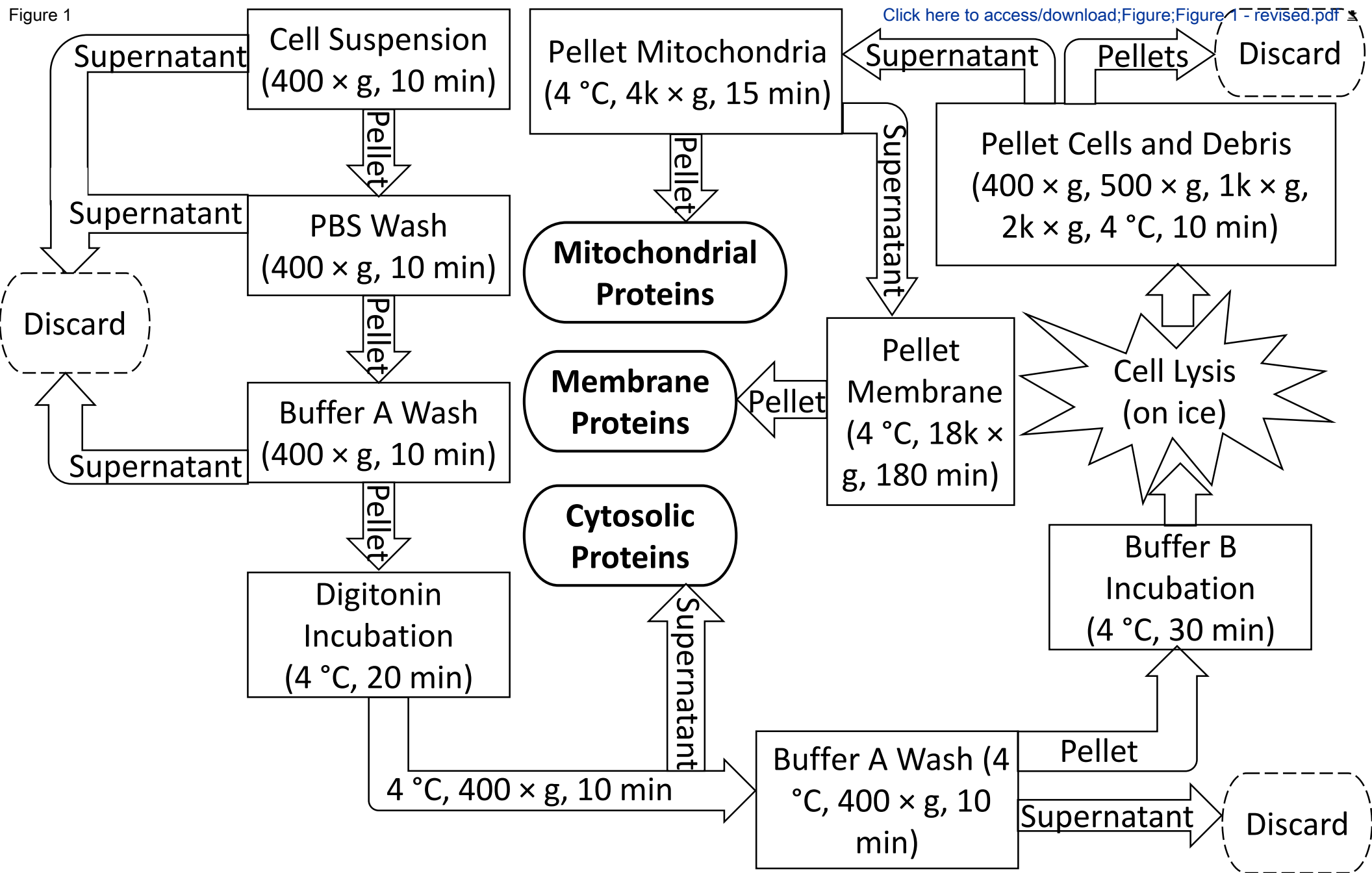

Figure 2

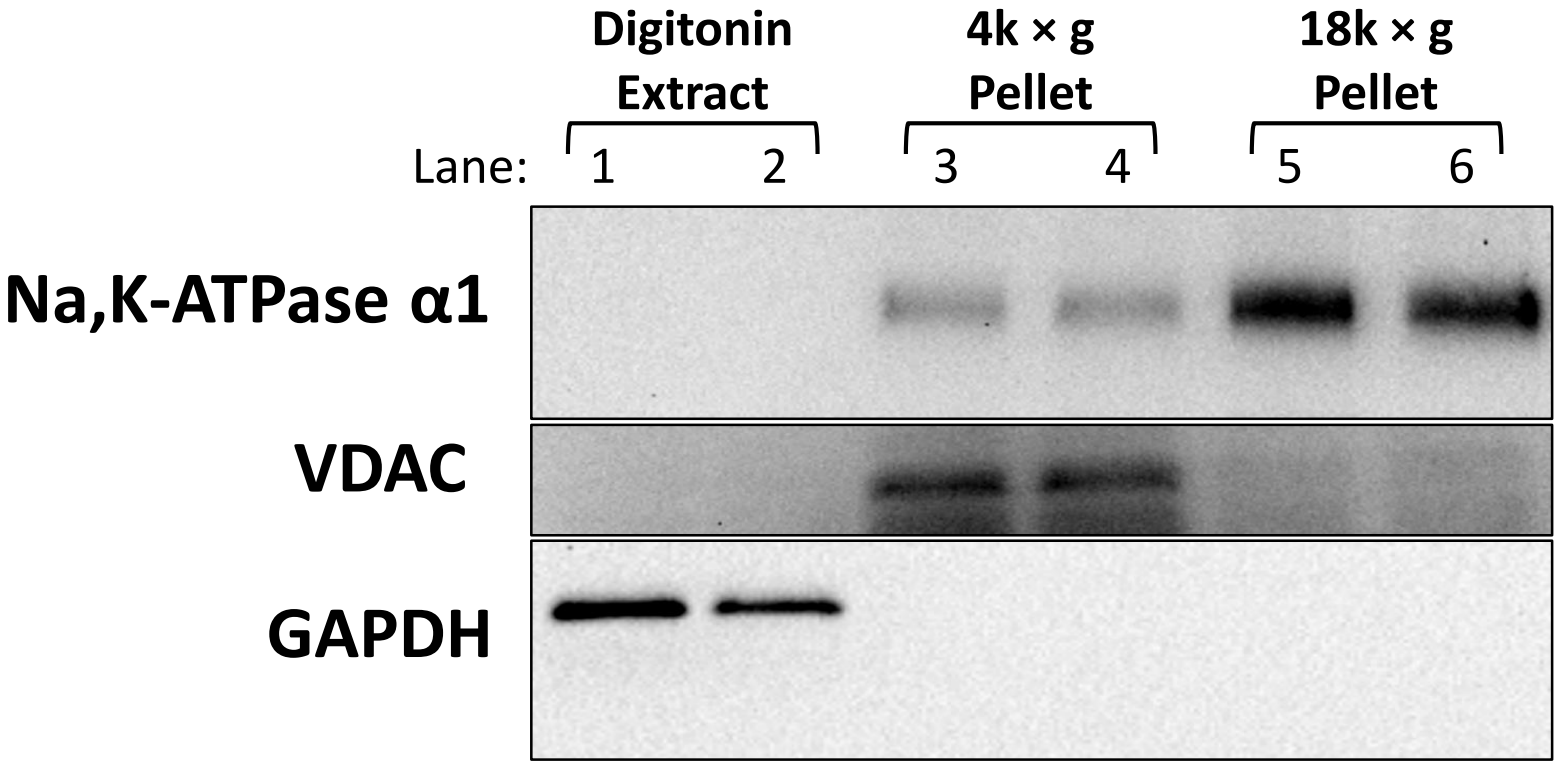

Figure 3

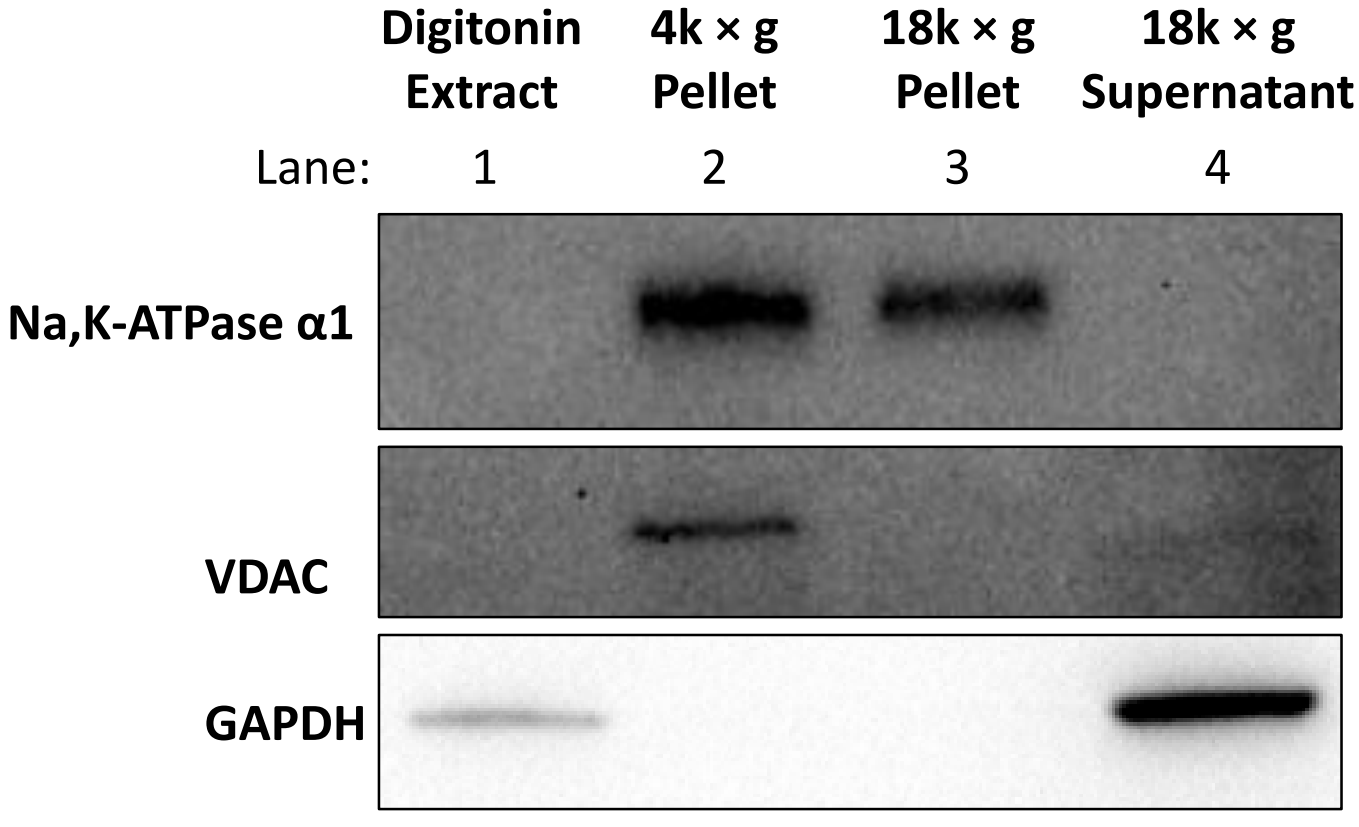

Figure 4

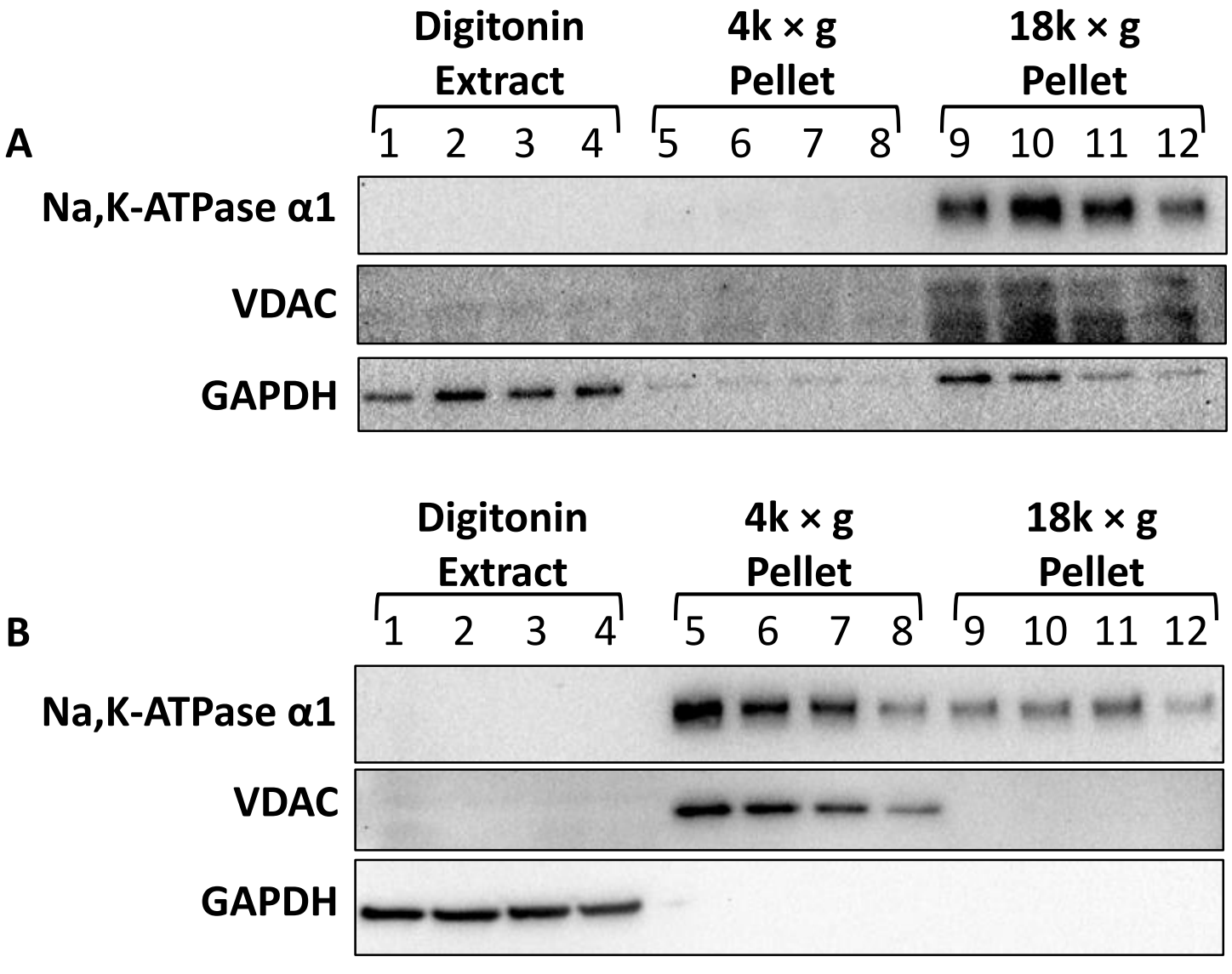

Table 1

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Name
Buffer A
Lysis Buffer B
Sample Buffer
Digitonin
Phenylmethanesulfonyl fluoride
Protease Inhibitor Cocktail
Sodium Orthovanadate

Composition (stock concentration)

NaCl (150 mM) and HEPES (50 mM)

HEPES (20 mM), KCl (10 mM), $MgCl_2$ (2 mM), EDTA (1 mM), EGTA (1 mM), Mannitol (210 mM) and Sucro

Sodium dodecyl sulfate (0.1%) in Tris-buffered Saline

Digitonin (250 µg/ml) in deionized water

Phenylmethanesulfonyl fluoride (100 mM) in 100% ethanol

Varies (See manufacturer product sheet)

Sodium Orthovanadate (500 mM) in deionized water

Final Concentration

1X (same as stock)

1X (same as stock)

1X (same as stock)

25 µg/ml

1 mM

1X

1 mM

Table 2

Protocol Step	Critical Factor
Cell Preparation	Cell concentration
	Pre-processing of cells
Cytosolic Protein Isolation	Digitonin Concentration
	Post-Extraction Wash
Mitochondrial Isolation	Cell Lysis

Debris Removal

Post-Isolation Wash

Membrane Isolation Centrifugation Time

Explanation

The optimal concentration of cells that this method can process must be determined empirically for the cell type being worked with in order to obtain the best results.

Cells must be in suspension for the fractionation procedure, this requires detaching adherent cells from culture surfaces or homogenizing tissues.

The optimal concentration of digitonin must be determined to avoid lysis of cells while still allowing cytosolic protein extraction through pore formation.

Removal of digitonin and cytosolic proteins from permeabilized cells must be performed to avoid cross contamination.

Lysis methods must be thorough to release cellular contents, while maintaining mitochondrial integrity for subsequent isolation.

Intact cells and larger fragments must be removed from the homogenate following lysis to avoid contaminating mitochondrial samples.

Mitochondrial samples should be washed thoroughly to remove contaminating debris that may pellet with them.

Centrifugation times may need to be extended to increase yield of membrane fraction sample.

Potential Issues	Possible Solutions
Highly concentrated cell suspensions may result in inefficient lysis, leading to low yields of mitochondria and membrane fractions.	Perform initial procedure with a range of cell concentrations to determine best results.
Inefficient processing may result in low fraction yields, cross contamination between subcellular fractions or other unexpected results.	Ensure that the method employed results in sufficient cells for the procedure. Count cells in suspension to determine concentration after processing and adjust accordingly.
If the method employed compromises the plasma membrane, premature lysis may occur, resulting in cross contamination of fractions.	Ensure that plasma membrane integrity is maintained during cell harvest by using methods that avoid damaging the cell. Verify membrane integrity by examination under a microscope with the inclusion of a membrane impermeable dye (such as Trypan blue).
High concentrations of digitonin may lead to membrane rupture, cell lysis and contamination of the cytosolic fraction. Suboptimal concentrations will result in inefficient extraction of cytosolic proteins and possible cross contamination of subsequent fractions.	The concentration of digitonin should be decreased if excessive cell lysis is observed. A small cell pellet obtained following digitonin incubation may indicate membrane rupture and cell lysis.
Failure to wash cells sufficiently after cytosolic extraction may result in carry over of cytosolic proteins to other fractions.	Additional washes with Buffer A following digitonin incubation will dilute digitonin and remaining cytosolic proteins.
Mechanical lysis of cells may be inefficient, leaving cells intact and resulting in low yields of mitochondrial proteins.	The amount of force needed to lyse cells and release mitochondria must be determined empirically for different cell types. Large pellets obtained after the lysis step (as well as small mitochondrial and membrane pellets) may indicate suboptimal lysis. Increase the amount of force (pestle strokes, needle passages, etc.) to minimize the post lysis pellet.

Too much mechanical force may lyse mitochondria, contaminating the plasma membrane fraction with mitochondrial membrane proteins.	Decrease the amount of force if mitochondrial protein markers are found in membrane samples.
Mitochondrial samples may become contaminated with cytoplasmic components or plasma membrane proteins if debris and intact cells are not removed prior to pelleting mitochondria.	Increase the number of low speed centrifugation steps prior to the mitochondrial isolation spin. If mitochondrial yield is low, it may be necessary to save pellets from low speed spins and check via western blot for mitochondrial markers.
Cell fragments may aggregate and associate with mitochondria, leading to cross contamination of cytoplasmic or membrane proteins.	Ensure that pellets are sufficiently washed with buffer to remove contaminants.
Depending on the total number of processed cells and the efficiency of cell lysis, membrane fraction sample yield may be low.	Increasing the centrifugation time may improve yield of plasma membrane fraction. While the suggested time is sufficient for a large starting quantity of cells, longer times may be necessary for smaller quantities.

Name of Material/ Equipment	Company	Catalog Number
Digitonin	TCI Chemicals	D0540
D-Mannitol	Sigma-Aldrich	M4125
Dounce homogenizer	VWR	22877-282
end-over-end rotator	Barnstead	N/A
ethylene glycol-bis(β-aminoethyl ether)-N,N,N',N'-tetraacetic acid (EGTA)	Alfa Aesar	J61721
Ethylenediaminetetraacetic acid (EDTA)	Sigma-Aldrich	E7889
GAPDH (14C10)	Cell Signalling Technologies	2118
HEPES	VWR	J848
KCl	Sigma-Aldrich	P9541
$MgCl_2$	Alfa Aesar	12315
Na, K-ATPase a1 (D4Y7E)	Cell Signalling Technologies	23565
NaCl	Sigma-Aldrich	793566
phenylmethanesulfonyl fluoride (PMSF)	VWR	M145
probe sonicator	Qsonica	Q125-110
Protease Inhibitor Cocktail, General Use	VWR	M221-1ML
refrigerated centrifuge	Beckman-Coulter	N/A
Sodium dodecyl sulfate (SDS)	VWR	227
sodium orthovanadate (SOV)	Sigma-Aldrich	450243
Sucrose	Sigma-Aldrich	S0389
Tris-buffered Saline (TBS)	VWR	788
VDAC (D73D12)	Cell Signalling Technologies	4661

Comments/Description

For Cytoplasm Extraction
For Lysis buffer B
For Homogenization
For Cytoplasm Extraction

For Lysis buffer B
For Lysis buffer B
For detection of cytoplasmic fractions on western blot, dilution: 1:10000
For Lysis buffers A and B
For Lysis buffer B
For Lysis buffer B
For detection of plasma membrane fractions on western blot, dilution: 1:1000
For Lysis buffer A
For Cytoplasm Extraction and Homogenization Buffer
For Final Samples
For Cytoplasm Extraction

For Sample buffer
For Lysis buffers A and B
For Lysis buffer B
For Sample buffer
For detection of mitochondrial fractions on western blot, dilution: 1:1000

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Author(s): William D. McCaig, Payal S. Patel, and Timothy J. LaRocca

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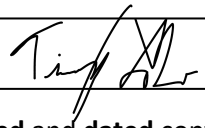
Signature:	
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Editorial comments:

1. Please take this opportunity to thoroughly proofread the manuscript to ensure that there are no spelling or grammar issues.

Thank you. We have proofread the entire manuscript.

2. Figures 3 and 4: These two figures are missing from the submission. Please upload these two figures individually to your Editorial Manager account as a .png, .tiff, .pdf, .svg, .eps, .psd, or .ai file.

Thank you for pointing this out. We have now uploaded these figures.

3. Please provide an email address for each author.

We have now provided email addresses for all authors in the text.

4. Please rephrase the Summary to clearly describe the protocol and its applications in complete sentences between 10-50 words: “Here, we present a protocol to …”

We have now rephrased the summary according to this suggestion.

5. Abstract: Please include an overview of the method and a summary of its advantages, limitations, and applications.

We have now rephrased the Abstract according to this suggestion.

6. 1.3: The Protocol should contain only action items that direct the reader to do something. Please either write the text in the imperative tense as if telling someone how to do the technique (e.g., “Do this,” “Ensure that,” etc.), or move the solutions, materials and equipment information to the Materials Table.

Thank you for this suggestion. We have now moved the solutions to Table 1. Our equipment information is in the Materials and Methods.

7. 2.1.3, 3.1.2, 3.1.5, 3.2.2, etc.: Please specify centrifugation parameters throughout the protocol.

We have now added the centrifugation parameters to the protocol.

8. 4.1.6: Please specify the volume (range) of Lysis Buffer B used.

We have now added the volume of lysis buffer B.

9. 5.1.3: Does the homogenate here refer to the supernatant obtained in step 5.1.2? Please specify.

Yes it does. Homogenate has now been renamed to supernatant (for clarity) and a reference to the originating step added.

10. 5.1.19: What is the desired final volume in this experiment? Please specify.

A suggested final volume range has now been added.

11. Please revise the Acknowledgements section to include any acknowledgments and all funding sources for this work.

Thank you for pointing this out. We have now added our funding source to the Acknowledgements

12. Please include a Disclosures section, providing information regarding the authors' competing financial interests or other conflicts of interest. If authors have no competing financial interests, then a statement indicating no competing financial interests must be included.

Thank you for pointing this out. We now include a disclosures section and declare no conflict of interest.

Reviewers' comments:

Reviewer #1:

Manuscript Summary:
This manuscript reports the use of a method for cell fractionation in the absence of high speed centrifugations or commercially available kits. This was accomplished by performing lysis, homogenization and centrifugations of U937 cells. Although this manuscript requires some minor revisions, it was well written. This study is important and can be a very useful method for those who lack funding for commercial kits or access to a high speed centrifuge.

Major Concerns:
None.

Minor Concerns:
Comment 1. I would recommend going through the protocol itself and modifying some of the numerical values to better represent significant figures. For example, in line 69, the authors call for the addition of 8.766 g of NaCl. Additionally, in line 73, the authors call for 0.746 g of KCl. These values would be hard to measure out. Thus, I would recommend rounding up and using less digits.

Values have been rounded for clarity.

Comment 2. There is an extra space between 100 and % in line 92.

Thank you. We have now corrected this.

Comment 3. There is also a period missing on the last sentence of line 243.

This has been corrected.

Comment 4. The authors seem to provide evidence of successful fractionation using this technique. I am just curious as to whether they have tried this method with any other cell lines and whether they had success. If so, I would recommend possibly adding this to the discussion and citing data not shown.

This fractionation procedure has only been attempted in the U937 cell line (in wild type and knockout lines). The text has been updated to clarify this.

Reviewer #2:

Manuscript Summary:
This paper describes a simple and reproducible method to isolate subcellular fractions, in particular the plasma membrane, mitochondria and cytosol. This method is of potential interest for a very large number of scientists using U937 cells and many other cell types.

Major Concerns:
Although this is my general opinion, I should mention that Authors normally perform an accurate revision of the manuscript, prior to its submission for publication. I therefore ask the Authors to include all the figures before submission of the revised paper. I could not find Figs 3 and 4 in the present version of the manuscript. Please also make sure to check on the Material and Methods section, in which I could find parts still highlighted in yellow.

Thank you for pointing this out. We do not know why there was an error in uploading these figures. Accordingly, figures 3 and 4 have been uploaded to the submission website. We included the yellow highlighting in our submission as it was our understanding that this was required by the publisher to indicate filmable content for the journal.

Minor Concerns:
Please also address the following issues:
-The title of the paper should be changed, since the method is employed for the isolation of three subcellular fractions.

The title has been altered, with the word “Complete” removed.

-Lysis Buffer A should be defined as Buffer A. It is not a lysis buffer.

We have changed this as suggested.

-Digitonin is used to "permeabilize" the cells and "isolate" cytosolic proteins. 3.2.1 and 3.2.2: please provide a more appropriate definition of cell pellet obtained after digitonin treatment.

The descriptor “digitonin-permeabilized” has been added to differentiate the cell pellets obtained after cytosolic extraction.

-Figure 1: Please provide more details on the scheme summarizing the steps of the protocol employed.

Figure 1 has been redesigned with more details, as suggested.

-A table with the composition of the buffers and solutions employed should be included. Consider also the possibility of including a table summarizing the critical steps of the procedure (i.e., the use of an appropriate digitonin concentration, assessment of the efficiency of homogenization, ecc..).

These tables have been added as suggested.

-Lines 317-318. The Authors state that the omission of SDS in the final buffer followed by density gradient centrifugation, leads to the isolation of intact mitochondria. Are these mitochondrial functional and suitable for their use in experiments measuring oxygen consumption, ROS formation, or other biological responses?

This statement has been removed. While the mitochondria should be intact and functional (based on similar isolation protocols), we have not tested this and removed this suggestion to avoid misleading readers.

- Please introduce more details on Western Blot procedure utilized.

Further details have been added to the Representative Results section indicating transfer method and membrane type.

Reviewer #3:

Manuscript Summary:
The submission " Complete Cell Fractionation in U937 Cells in the Absence of High Speed Centrifugation" by McCaig, Patel and LaRocca describes a protocol to isolate soluble, mitochondrial and membrane fractions from human cell culture. The protocol is well described, well detailed and seems to be suitable for publication in JOVE. I have a few specific comments that might improve the manuscript and that the authors should address in preparing their final manuscript.

Major Concerns:
In particular, I can't assess some of the claims of purity because figures 3 and 4 were omitted from the review copy of the manuscript I received. I think these panels would help because I can't tell how troubleshooting would work here in a case where the protocol wasn't properly fractionated. To this end, it would help if the authors described what steps are most critical for the separation to be successful. It is strongly implied (although not explicitly stated) that the total number of spins at a lower speed (e.g. 5.1.1 to 5.1.8) is that factor to ensure that the sample being worked with has contaminating fractions

removed from it. Is that true? It would be worth highlighting if the authors know or suspect that this is where substantial tweaks might have to be done by someone working with different samples.

We do not know why there was an error in uploading these figures. Figures 3 and 4 have been uploaded to the submission website. A table with critical steps for purification has been added for troubleshooting purposes and adaptation to other cell lines.

Minor Concerns:
I'd also like to see some discussion of cells types/tissues that this protocol isn't suited for. The authors describe it as working in U937 cells, but there isn't a great description of this cell line in the manuscript nor is it referenced. Have the authors ever tried this on other cells types or on intact tissues? Does it work when there is significant levels of connective tissue present? I don't think they need to test every possibility, but it would be good to explicitly state that this is really for in vitro passaged cells if that is the case.

We have not attempted this protocol with tissues or cell lines other than U937 cells. We have provided suggestions in the Discussion for the processing of adherent cells and tissues, however, these are only suggestions and we have not performed these experiments.

Line 44 - define "standard" here

This has now been defined in the text.

Line 313 - do the authors have a list of cell types they have used? Are there tissue/cell specific information they can offer here on successfully carrying out the protocol?

We have performed this protocol in U937 cells only. We have added text to reflect that we have only obtained results in the U937 cell line. We have also added suggestions for additional cell lines and tissues in the Discussion.

Line 319 - it seems a bit of an oversight to not probe for nuclear markers and nuclear marker purity here

This statement has been removed, as we have not probed for nuclear markers. The suggestion was based on similar published protocols, but we have no definitive evidence of this. The work for which this protocol was developed did not require analysis of the nuclear fraction.