Sucrose Gradient Separation Protocol

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CONTENT

I. REQUIRED REAGENTS AND EQUIPMENT

Reagents:

- n-dodecyl-β-D-maltopyranoside (Lauryl maltoside; MitoSciences MS910)
- Phosphate buffered saline, PBS (section VII)
- Sucrose solutions 15, 20, 25, 27.5, 30 and 35 %
- Double distilled water
- Protease inhibitor cocktail (section VII)
- 13 x 51 mm polyallomer centrifuge tubes (Beckman 326819)

Equipment:

- Swing-out compatible ultracentrifuge and rotor (e.g. Beckman SW50.1)
- pH meter, weighing balance and other standard lab equipment
- Laboratory benchtop microfuge
- Protein electrophoresis and Western blotting equipment

II. SAMPLE PREPARATION

The MitoSciences sucrose gradient separation procedure is a protein subfractionation method optimized for mitochondria. It can be used to reduce sample complexity facilitating large-scale proteomics efforts (Taylor *et al*, [1]Hanson *et al*). Also when this procedure was coupled to MitoSciences highly sensitive Western blotting antibodies it allows the detection of mis-assembled mitochondrial enzyme complexes in patient cell lines ([2]Hanson *et al*).

This method resolves a sample into at least 10 fractions. It is possible to separate solubilized whole cells into fractions of much lower complexity but when analyzing already isolated mitochondria the fractions are even more simplified. Details of mitochondrial isolation can be found at www.mitosciences.com/PDF/mitos.pdf

The total amount of OXPHOS complexes in mitochondrial samples/ cell samples varies greatly between species and tissues. Therefore it is **highly** recommended that, during the experimental planning steps, an estimation of the total amount of protein in the users sample is made. In this way the appropriate detection strategy can be employed. Table I suggests detection strategies based amount of starting material.

Table 1. *Suggested detection methods by amount starting material.*

Starting mitochondria applied to the gradient	**Recommended detection strategy**
5 mg +	Gel staining with coomassie
0.5 mg +	Gel staining with silver/sypro ruby
< 0.5 mg	Western blotting with MitoSciences mAbs

Taylor SW, Fahy E, Zhang B, Glenn GM, Warnock DE, Wiley S, Murphy AN, Gaucher SP, Capaldi RA, Gibson BW, Ghosh SS. Characterization of the human heart mitochondrial proteome. Nat Biotechnol. 2003 Mar; 21(3): 239-40.

[1] Hanson BJ, Schulenberg B, Patton WF, Capaldi RA. A novel subfractionation approach for mitochondrial proteins: a three-dimensional mitochondrial proteome map. Electrophoresis. 2001 Mar; 22(5): 950-9.

[2] Hanson BJ, Carrozzo R, Piemonte F, Tessa A, Robinson BH, Capaldi RA. Cytochrome c oxidase-deficient patients have distinct subunit assembly profiles. J Biol Chem. 2001 May 11; 276(19): 16296-301.

III. SAMPLE SOLUBILIZATION

The sucrose gradient separation technique detailed in this manual is designed for an initial sample volume of up to 0.5 ml at 5 mg/ml protein. Therefore 2.5 mg or less of total protein should be used. For larger amounts, multiple gradients can be prepared or larger scale gradients are also possible, see section VIII.

The sample should be solubilized in a non-ionic detergent. It has been determined that at this protein concentration mitochondria are completely solubilized by 20 mM **n-dodecyl-β-D-maltopyranoside** (1% w/v **lauryl maltoside**). The key to this solubilization process is that the membranes are disrupted while the previously membrane embedded multisubunit OXPHOS complexes remain intact, a step necessary for this density based sucrose separation procedure described here*.

• To a mitochondrial membrane suspension at 5 mg/ml protein in PBS add lauryl maltoside to a final concentration of 1 %. Mix well and incubate on ice for 30 minutes. Centrifuge at 72 000 g for 30 minutes. The Beckman Optima benchtop ultracentrifuge is recommended for small sample volumes** (however at a minimum a benchtop microfuge on maximum speed should suffice, which is usually around 16 000 g). The supernatant is collected and the pellet discarded. Add a protease inhibitor cocktail (see section VII) and keep the sample on ice until centrifugation is performed. Note: with samples very rich in mitochondria the cytochromes in complexes III and IV may give this supernatant a brown color, which is useful when checking the effectiveness of the separation in section V.

* One important exception is the pyruvate dehydrogenase enzyme: In order to isolate PDH at a protein concentration of 5 mg/ml mitochondria, the required detergent concentration is only 10 mM (0.5 %) lauryl maltoside. ** The PDH enzyme should also be centrifuged at lower speeds, a centrifugal force of 16 000 g is maximum for the PDH complex.

IV. SUCROSE GRADIENT PREPARTION

A discontinuous sucrose density gradient is prepared by layering successive decreasing sucrose densities solutions upon one another. The preparation and centrifugation of a discontinuous gradient containing sucrose solutions from 15-35 % is described in detail in this manual. This gradient gives good separation of the mitochondrial OXPHOS complexes (masses ranging from 200 kDa to 1000 kDa). However this setup can be modified for the separation of a particular complex or for the separation of larger amounts of material (the details of optimization steps and considerations are given in section VIII).

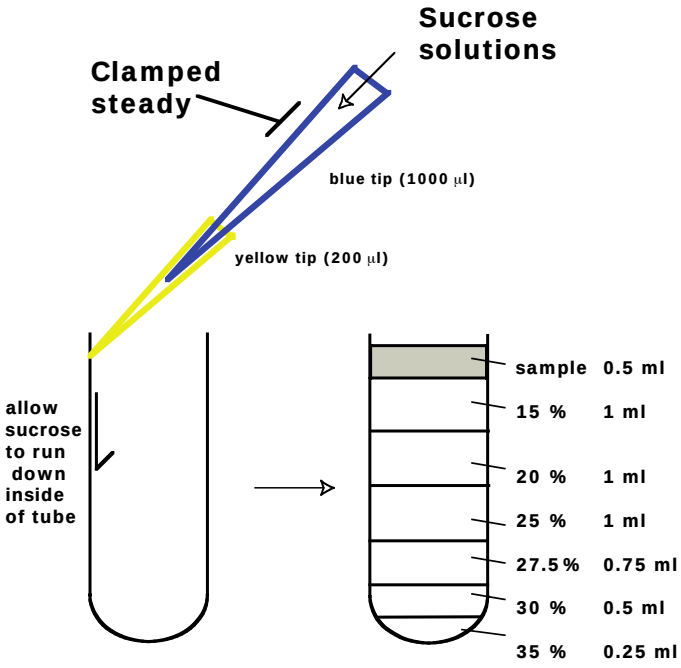


Figure 1. Setup for sucrose gradient preparation

• **15-35 % sucrose density gradient**. The preparation of these sucrose solutions is described in section VII. The gradient is prepared by layering progressively less dense sucrose solutions upon one another; therefore the first solution applied is the 35 % sucrose solution. A steady application of the solutions yields the most reproducible gradient therefore the setup described above in Figure 1 is recommended. Firstly a Beckman polyallomer tube is held upright in a tube stand. Next a yellow (200 µl) pipettor tip is placed on the end of a blue (1000 µl) pipettor tip. Both snugly fitting tips are held steady by a clamp stand and the end of the yellow tip is allowed to make contact with the inside wall of the tube as shown below. Now sucrose

solutions can be placed inside the blue tip and gravity will feed the solutions into the tube slowly and steadily, starting with the 35 % solution (the volumes of this and the other solutions are shown in Table II). Note: if the solutions fail to feed down through the tips and into the tube a small amount of positive air pressure can be applied to the blue tip to start the flow. This is done by gently tapping on the wide end of the blue tip. Once the 35 % solution has drained into the tube, the 30 % solution can be loaded into the blue tip which will then flow down the inside of the tube and layer on top of the 35 % solution. This procedure is continued with the 27.5 %, 25 %, 20 % and 15 % respectively. There should now be enough space left at the top of the tube upon which to pipette the 0.5 ml sample of solubilized mitochondria described in section III.

	Solution	**Volume**
1 (top)	Sample	0.5 ml
2	15 % sucrose	1 ml
3	20 % sucrose	1 ml
4	25 % sucrose	1 ml
5	27. 5 % sucrose	0.75 ml
6	30 % sucrose	0.5 ml
7 (bottom)	35 % sucrose	0.25 ml
		5 ml total

Table II. Volumes of solutions used.

V. CENTRIFUGATION AND ELUTION OF FRACTIONS

Once the sucrose gradient is poured discrete layers of sucrose should be visible. Having applied the sample to the top of the gradient the tube should be handled and loaded into the rotor very carefully. Centrifugation should begin as soon as possible. However all centrifugation procedures require a balanced rotor therefore another tube containing precisely the same mass must be generated. In practice this means 2 gradients must be prepared although the second gradient need not contain an experimental sample but could contain 0.5 ml water in place of the 0.5 ml protein sample.

- The polyallomer tubes should be centrifuged in a swinging bucket SW 50.1 type rotor (Beckman) at 37 500 rpm (RCF_{av} 132 000 x g) for 16 hours 30 minutes at 4 ^{o}C with an acceleration profile of 7 and deceleration profile of 7.

- Immediately after the run the tube should be removed from the rotor, taking great care not to disturb the layers of sucrose. When separating a sample rich in mitochondria, discrete colored protein layers might be observable. Most often these are Complex III (500 kDa – brown color) approximately 10 mm from the bottom of the tube and Complex IV (200 kDa – green color) 25 mm from the bottom of the tube. In some circumstances additional bands can be observed. These are the other OXPHOS complexes.

- For fraction collection the tube should be held steady and upright by a clamp stand. A tiny hole should be introduced into the very bottom of the tube using a fine needle. The hole should be just big enough to allow the sucrose solution to drip out at approximately 1 drop per second. Fractions of equal volume are then collected in eppendorf tubes below the pierced hole. A total of 10 x 0.5 ml fractions are appropriate however collecting more fractions which are thus smaller in volume is also possible e.g. 20 x 0.25 ml fractions. The fractions can now be stored at – 80^{o}C

V. SAMPLE ANALYSIS

Fractions collected can now be resolved by electrophoresis. Resolved proteins should be detected by the method chosen in Table I above. Optimized protocols for electrophoresis, gel staining and Western blotting can be found at www.mitosciences.com/PDF/western.pdf
As described in these protocols samples are first solubilized in SDS-PAGE sample buffer, see section VII. Prior to loading the sample can be reduced by 50 mM DTT or 1 % β-mercaptoethanol. Another optional step is heating of the sample, which can be done at 95°C for 5 minutes or at 37°C for 30 minutes prior to loading onto the gel. These steps may increase the resolution of protein bands and also reduce the complexity of the sample by breaking any disulfide bonded proteins.

A typical example of fractions taken from a sucrose gradient of solubilized heart mitochondria are shown below. 10 µl of each fraction was resolved by SDS-PAGE before coomassie staining.

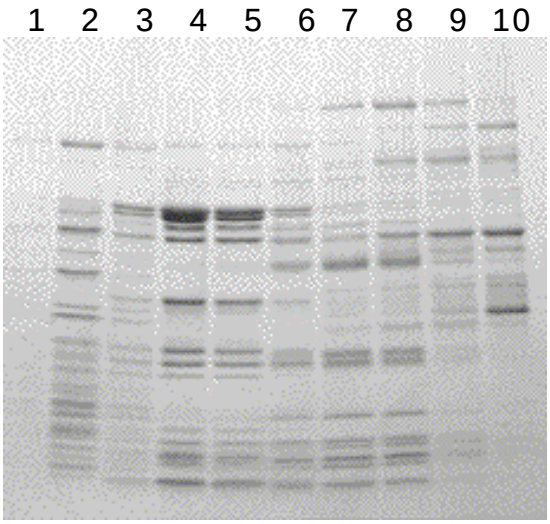


Figure 2. A typical sucrose gradient separation of mitochondria showing enrichment of OXPHOS Complex I (predominates in lane 2), Complex II (lane 8), Complex III (lane 4 & 5), Complex IV (lane 7 and 8), and Complex V (lane 4).

VI. BUFFER RECIPES

Phosphate buffered saline solution (PBS)
1.4 mM KH_2PO_4
8 mM Na_2HPO_4
140 mM NaCl
2.7 mM KCl, pH 7.3

Lauryl maltoside stock
200 mM n-dodecyl-β-D-maltopyranoside (10 % w/v lauryl maltoside) (Anatrace D310S)

Protease inhibitor stocks (each is 1000 x)
1 M phenylmethanesulfonyl fluoreide (PMSF) in acetone (Sigma L7626)
1 mg/ml leupeptin (Sigma L2884)
1 mg/ml pepstatin (Sigma P4265)

Sucrose solutions
To six 15 ml tubes polypropylene centrifuge tubes (Corning)
add 1.5g, 2g, 2.5g, 2.75g, 3g and 3.5 g sucrose.
Fill each tube to 10 ml with 50 mM Tris. HCl pH 7.5, 1 mM EDTA, 0.05 % lauryl maltoside.
Turn the tubes on a rotator for approximately 20 minutes until all the sucrose has dissolved.

2x SDS page sample buffer
20 % glycerol
4 % SDS
100 mM Tris pH 6.8
0.002 % Bromophenol blue
optional – 100 mM dithiothreitol

VII. OPTIMIZATION STEPS AND GENERAL TIPS

Protein determination

Each fraction should be 500 μl (or less if greater than 10 fractions were collected). The protein content within each fraction can be determined by protein assay, we recommend the BCA or micro BCA methods (Pierce).

Protein precipitation for concentration and/or sucrose removal

The proteins in the sample can be precipitated for concentration and sucrose removal however precipitated proteins will be denatured. We recommend the Chloroform/methanol precipitation method. Briefly add 600 μl fraction methanol to a 150 μl of a fraction. After thorough mixing 150 μl chloroform is added. Vortex then add 450 μl water, vortex again (appears cloudy white) and centrifuge immediately for 5 minutes at full speed in a microfuge. A white disc of protein should form between the organic layer (bottom) and the aqueous layer (upper). Discard the upper aqueous layer. Add 650 μl of methanol to the tube and invert 3 times. Spin for 5 minutes at full speed in a microfuge. Remove all liquid and allow the pellet to air dry. The precipitated protein pellet can be taken up in SDS PAGE sample buffer for electrophoresis and/or protein assay.

Other sucrose gradient formulation

The sucrose gradient concentrations and number of layers can be altered when optimizing for the separation of a particular enzyme complex. For example to improve the separation of Complex I (the largest complex) proportional volume of the 27.5 %, 30 % and 35 % sucrose solutions should be increased. An example of such a gradient would be 500 μl each of a 37.5% 35%, 32.5%, 30%, 27.5%, 22.5%, 20%, 17.5%, and 15% sucrose solution and a 500 μl sample makes the total volume of 5 ml.

Larger sucrose gradients are possible– an example

Gradients larger than 5 ml can be prepared for separation of starting samples greater than 2.5 mg protein (i.e. 500 μl sample at 5 mg/ml). However larger tubes and centrifuge rotors are necessary.

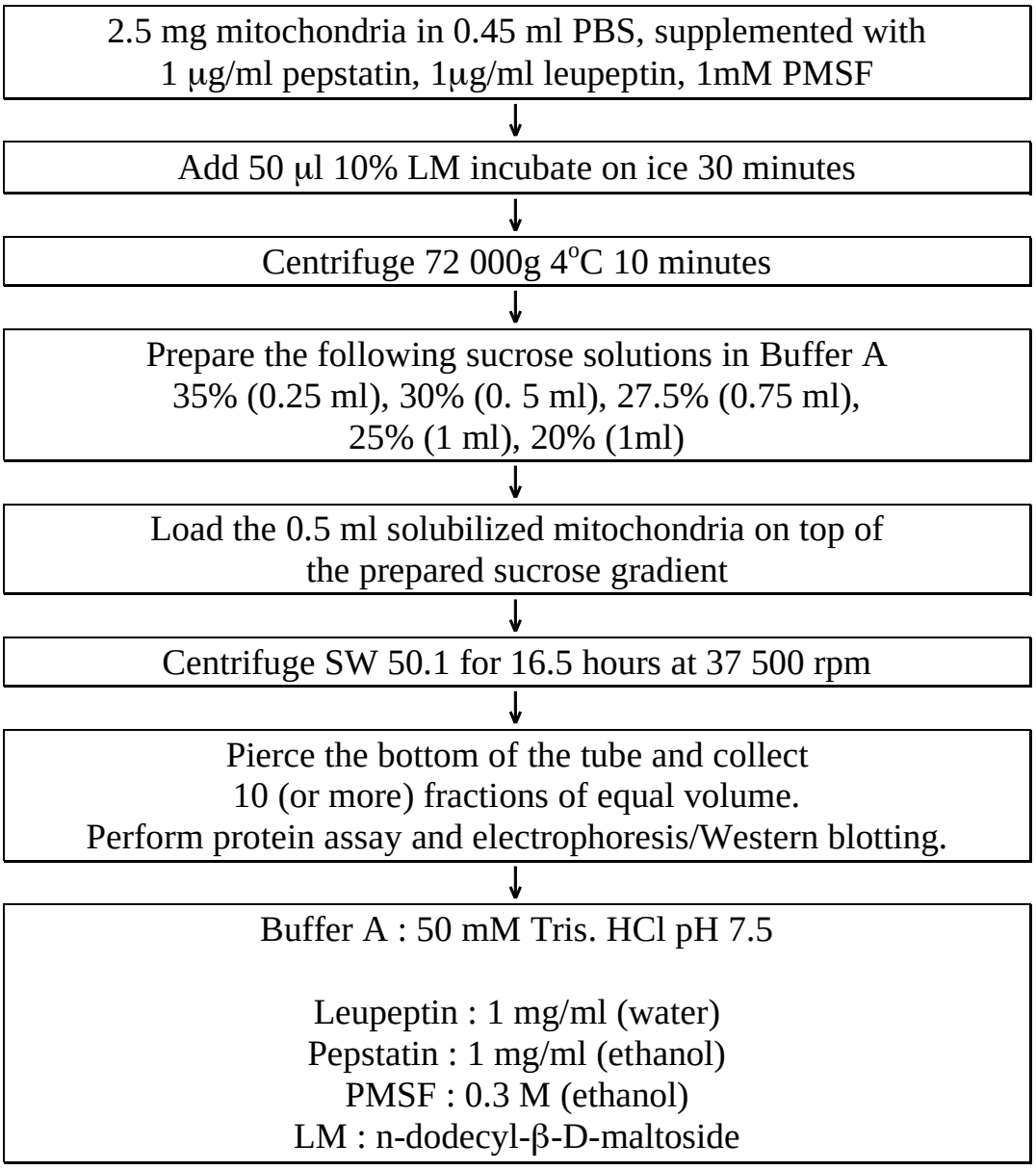
As an example 40 mg of mitochondria can be resolved by a 55-25% 41 ml sucrose gradient. This is made by layering sucrose solutions of 55% (5 ml), 50% (5 ml), 35% (10 ml), 30% (10 ml), 25% (3 ml), and finally on top 8 ml of solubilized mitochondria (which is the supernatant from 8 ml of mitochondria (5 mg/ml) solubilized by 1% lauryl maltoside and centrifuged 30 min 72 000 g as described above in section III). This gradient should be centrifuged at 37 000 rpm (113 000 RCF_{av}) in a VTi50 type rotor for 17 hours at 4^{o}C with acceleration profile 9 and deceleration profile 9.

VIII. TROUBLESHOOTING GUIDE

No bands appear after centrifugation	In order to see resolved bands the sample must be rich in mitochondria. Prior to centrifugation the sample might appear colored brown. If not then the mitochondrial content may be lower and therefore the more sensitive methods of gel staining/Western blotting detection should be chosen.
	The bands may have diffused after centrifugation before fraction collection
	The sucrose layers may have been disturbed
Weak or no gel staining signal	Isolate mitochondria from the sample
	Increase the amount of sample
	Silver stain the gel
	Western blot the gel and detect with MitoSciences antibodies
Non-specific bands when Western blotting	Isolate the mitochondria to higher purity
	Add a reducing agent to the eluted sample e.g. DTT
	Heat the eluted sample at 95 ^{o}C for 5 minutes before loading

IX. FLOW CHART

This guide is for quick reference only. Be completely familiar with the previous details of this document before performing the assay.



X. NOTES

www.mitosciences.com
Phone: 1 800 910-6486
Fax: (541) 284-1801