OptiPrep™ Application Sheet S07

Differential centrifugation of homogenates

- To access other Application Sheets referred to in the text return to the Subcellular Membranes Index; key Ctrl "F" and type the S-Number in the Find Box.

1. Background

The employment of differential centrifugation to prepare crude fractions of subcellular particles from homogenates is often a necessary first step to a subsequent purification of one or more particles on a density gradient. Buoyant density gradient purification of peroxisomes or lysosomes for example is almost invariably carried out on a light mitochondrial fraction so as to eliminate smaller particles that may have similar densities. Unless they are first removed, large rapidly sedimenting particles in homogenates may also disturb shallow gradients designed to fractionate small low density microsomes

This Application Sheet describes the use differential centrifugation to fractionate a mammalian liver homogenate but similar methods should be applicable to all mammalian tissues and cultured cells. Refs 1-5 describe many of these procedures in more detail. Although the homogenization methods for other cells such as yeast are rather different to those for mammalian cells, the subsequent processing of the homogenate by differential centrifugation is probably rather similar. The processing of homogenates from plant tissues is rather more specialized and is not covered in this text.

2. Homogenization medium

The solutions used for homogenization, washing and resuspension of the pellets, depend upon the organelle to be purified. They were developed for work with rat liver and other soft tissues and generally contain sucrose as the osmotic balancer.

A. General Purpose: 0.25 M sucrose, 1 mM EDTA, 20 mM Hepes-KOH, pH 7.4

B. Nuclei: As General Purpose but replace 1 mM EDTA with 25 mM KCl, 5 mM $MgCl_2$.

C. Peroxisomes: Add 0.1% (v/v) ethanol to Solution A.

D. Mitochondria: 0.2 M mannitol, 50 mM sucrose, 1 mM EDTA, 20 mM HEPES-KOH, pH 7.4.

Keep the following stock solutions at 4°C:

500 mM Hepes (free acid)	11.9 g per 100 ml water.
100 mM EDTA (Na_2•$2H_2O$)	3.72 g per 100 ml water
1 M KCl	7.45 g per 100 ml water
1 M $MgCl_2$•$6H_2O$	20.3 g per 100 ml water

Solution A: Dissolve 17 g sucrose in 100 ml water; add 2 ml of EDTA stock and 8 ml of Hepes stock; adjust to pH 7.4 with 1 M KOH and make up to 200 ml.

Solution B: As Solution A but replace EDTA with 1.0 ml of the $MgCl_2$ stock and 5.0 ml of the KCl stock

Solution C: As Solution A but replace 17 g sucrose with 7.28 g mannitol and 3.42 g sucrose

Many cultured cells can also be homogenized in the General Purpose medium or some other similar isoosmotic medium, see **Application Sheet S06**.

- If the homogenization has been carried out in a hypoosmotic medium, then this should be adjusted to the recommended concentration of sucrose and other additives as soon as possible after homogenization is complete.
- It is very important to check by phase contrast microscopy that the homogenization process has been successful in breaking at least 90% of the cells before attempting to carry out any differential centrifugation.

3. Centrifugation Equipment

- To achieve the best resolution and recovery of a specific subcellular particle, a fixed-angle rotor should be used for all differential centrifugation. The shorter the sedimentation path length of the rotor, the better will be the resolution and recovery. For a full explanation of the choice of rotor see refs 6 and 7.

Swinging-bucket rotors tend to have long path lengths and the only merit of their use is that the pellet, being formed at the bottom of the tube, is rather more stable and less likely to slide during decantation.

- ♦ After centrifugation in a fixed-angle rotor always decant the supernatant "away" from the pellet or use a syringe and metal cannula to harvest each supernatant.

4. Protocol

Carry out all operations at 0-4°C and all solutions should be pre-cooled on ice

1. Prepare the homogenate according to one of the methods described in **OptiPrep™ Application Sheets S05 or S06.**

2. If the nuclear pellet is to be processed, filter the homogenate through four layers of cheesecloth or fine nylon mesh (pore size 75 μm) to remove any unbroken cells and connective tissue. This filtration is not normally necessary for cultured cells.

3. Pellet the **nuclear fraction** by centrifugation at $1000g_{av}$ for 10 min (see Notes 1-5).

4. Pellet the **heavy mitochondrial fraction** by centrifuging the post-nuclear supernatant at $3,000g_{av}$ for 10min (see Notes 2-5).

5. Pellet the **light mitochondrial fraction** by centrifugation of the heavy mitochondrial supernatant at $15,000$-$17,000g_{av}$ for 10 min (see Notes 2-5).

6. Pellet the **microsomal fraction** by centrifuging the light mitochondrial supernatant at $100,000g_{av}$ for 45 min (see Note 5).

7. Resuspend all pellets in the appropriate medium by gentle homogenization with a loose-fitting Dounce homogenizer (approx. 0.5 mm clearance) to ensure complete dispersion of the pellets.

5. Notes

1. Centrifugation of the nuclear pellet is very often carried out in a swinging-bucket rotor rather than a fixed-angle rotor. In this case, the nuclei and debris are so large and rapidly sedimenting, compared to the other particles, that the long path length of such a rotor is not a real disadvantage.
2. To improve the recovery of more slowly-sedimenting particles and increase the purity of the differential centrifugation fractions it may be necessary to wash the pellets, in which case the resuspended pellets should be adjusted to about half of the volume of the homogenate and then recentrifuged at the same speed and time. The two supernatants are then combined prior to centrifugation at the next step.
3. Sometimes this washing is extended to three or more cycles of resuspension and recentrifugation; e.g. for the purification of mitochondria from the 3000g pellet.
4. Although the washing procedure can produce gains in recovery and/or purity of particles, it should always be a primary aim to minimize the amount of pelleting and resuspending as this causes progressive fragmentation of particles. It is also very time consuming.
5. The composition and analysis of the pellets are described in Sections 6 and 7

6. Composition of the pellets

The composition of the various fractions produced by differential centrifugation have been well defined for commonly used tissues such as mammalian liver, but for many cultured cells the distribution of the various membrane particles is rather less clear.

The **Nuclear Pellet** contains, in addition to nuclei, mitochondria, sheets of plasma membrane (if present) and, if the homogenate has not been filtered, unbroken cells and debris (including connective tissue). Formation of this pellet is sometimes carried out at 500g rather than 1000g.

The **Heavy Mitochondrial Pellet** contains predominantly, mitochondria with rather few contaminants and is a common source of these organelles for respiratory studies. Minor components such as lysosomes, peroxisomes, Golgi membranes and various membrane vesicles are present largely because of entrapment during the pelleting process. Some plasma membrane fragments may also be present. These contaminants can be reduced by repeated washing.

The **Light Mitochondrial Pellet** contains mitochondria, lysosomes, peroxisomes, Golgi membranes and some endoplasmic reticulum. Of all differential centrifugation fractions it is the most variable in terms of the actual centrifugation parameters used: g-forces of 15-20,000g and times of 10-20 min are the most common. Some methods are designed to maintain the Golgi membranes in their "stacked" form so that they sediment at much lower g-forces (see ref 3 for more information)

The **Microsomal Pellet** is rather better defined and contains only membrane vesicles. Some of those vesicles will have been present in the cell before homogenization (e.g. endosomes, secretory vesicles and vesicles from the *trans*-Golgi network), others from the plasma membrane, Golgi and smooth and rough endoplasmic reticulum, will have been produced by the homogenization procedure.

7. Analysis of pellets

Although the operator may be interested only in processing one of the pellets, it is nevertheless important to analyze all of the pellets for chemical and enzyme markers (Table 1) and protein. This will allow determination of the recovery, not only of the particle of interest but also of contaminants, which may be difficult to remove. Analysis of the cytosolic fraction (100,000g supernatant) should always be included; this not only permits complete and valuable “book-keeping” of organelle markers, it can also give information on possible disruption to organelles and consequent release of organelle contents during the homogenization procedure.

Table 1 Chemical and enzyme markers for subcellular membranes

Subcellular Particle	Marker*	Comment
Nucleus	DNA	
Mitochondria	Succinate dehydrogenase	Glutamate dehydrogenase is another commonly used marker
Lysosomes	Acid phosphatase, β-galactosidase	Other hydrolytic enzymes, e.g. β-*N*-acetylglucos-aminidase, may be used
Peroxisomes	Catalase	Method using titanium oxysulphate much easier to execute than one using $KMnO_4$
Endoplasmic reticulum (ER)	NADPH-cytochrome c reductase, rotenone-insensitive NADH-cytochrome c reductase	Glucose-6-phosphatase is only a reliable marker for liver and kidney ER.
Rough ER	As ER plus RNA	
Golgi	UDP-galactose galactosyl transferase	Method using ovalbumin as galactose acceptor is easiest to execute. Properly a marker only for the *trans*-Golgi.
Plasma membrane	5’-nucleotidase, Na^+/K^+-ATPase, leucine aminopeptidase, alkaline phosphatase	Only 5’-nucleotidase and alkaline phosphatase are reliable markers for cultured cells. Plasma membrane domain specificity of these markers is common in morphologically polarized cells.

* See ref 8

For more information on the analysis of membrane fractions see Application Sheet S09

8. References

1. Evans, W. H. (1992) *Isolation and characterization of membranes and cell organelles* In: Preparative Centrifugation - A Practical Approach (ed Rickwood, D.) Oxford University Press, Oxford, UK, 233-270

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3. Graham, J. M. (1997) *Homogenization of tissues and cells* In: Subcellular Fractionation - a practical approach (ed Graham, J. M. and Rickwood, D.), Oxford University Press, Oxford, UK pp 1-29
4. Hinton, R. H. and Mullock, B. M. (1997) *Isolation subcellular fractions* In: Subcellular Fractionation - a practical approach (ed Graham, J. M. and Rickwood, D.), Oxford University Press, Oxford, UK pp 31-69
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