OptiPrep™ Application Sheet S14a

Purification and analysis of mammalian mitochondrial fractions

- **This Application Sheet presents the options available for resolution of mitochondria (and other organelles) from mammalian tissues and cultured cells. Reference List RS03 lists all the published papers, reporting the use of iodixanol gradients, according to tissue/cell type**

1. Introduction

The choice of homogenization procedure, pre-gradient differential centrifugation and type of gradient can all contribute to the successful purification of mitochondria. Almost all the methodology was developed from work with rat or mouse liver, translation to other tissues and cells often requires changes to one or more protocol parameters.

2. Homogenization media

There are many examples of the use of a standard isoosmotic medium such as 0.25 M sucrose, 1 mM EDTA, buffered with 10-25 mM Tris to pH 7.0-7.8 for a variety of tissues and cells; the Tris often being replaced by the more organelle-friendly HEPES or Tricine. Functional studies with mitochondria may benefit from the inclusion of mannitol in the homogenization medium (HM) and there are many examples in which 0.25 M sucrose is replaced by 190-220 mM mannitol + 50-110 mM sucrose (e.g. refs 1-7). EGTA (at 1 mM) may replace the EDTA [1,4,7,8] or supplement the EDTA [9]. Virtually all media for tissues conform broadly to these recipes. An excellent description of the homogenization of bovine heart can be found in ref 10. Note that all media are routinely supplemented with a wide range of protease inhibitors. These isoosmotic media may also be used for cultured cells but there is a much more diverse range of medium composition, sometimes tailored to the cell type and/or to the subsequent analysis. For example the sucrose/mannitol medium for neonatal cardiac myocytes also included 2 mM taurine, 1 mM carnitine, 1 mM pyrophosphate and 1 mM calyculin [1,11-13]. Sometimes bovine serum albumin is included at 0.25-0.5% [8,12].

To achieve an effective and rapid homogenization some cultured cells must be swollen in a hypoosmotic medium, which, after cell lysis, is adjusted back to isoosmolality by the addition of a concentrated sucrose solution. Table 1 describes some of the variations. Sometimes $MgCl_2$ (2 mM final concentration) is added to protect the nuclei once homogenization is completed [17].

Table 1 Hypoosmotic homogenization media for cultured cells

Cell type	Homogenization medium	Ref #
Carcinoma (KB-V1)	150 µM $MgCl_2$, 10 mM KCl, 10 mM Tris-HCl, pH 6.7	14,15
HeLa	25 mM KCl, 1 mM EGTA, 10 mM HEPES-KOH, pH 7.5	16
Macrophages	100 mM sucrose, 1 mM EGTA, 10 mM HEPES-KOH, pH 7.4	17
MDCK transduced (carcinoma)	10 mM KCl, 1.5 mM $MgCl_2$, 1mM EGTA, 1 mM EDTA, 1mM DTT, 20 mM HEPES-KOH, PH 7.5	18

3. Differential centrifugation

A classical differential centrifugation scheme is shown in Figure 1. The *g*-force used to produce the nuclear pellet varies from 600 to 1000 *g* with times of 5-10 min. Values outside these ranges are rare, though only 200 *g* for 10 min was used for macrophages [17]. Occasionally the "heavy mitochondrial pellet" (HMP) is used as the gradient input [5,14,15,17,18], in which case the *g*-force is usually increased to 5000 *g*. The use of this pellet has the advantage of eliminating many of the peroxisomes and lysosomes that normally sediment only at the higher *g*-forces used to produce the "light mitochondrial pellet" (LMP), but there is a significant loss of

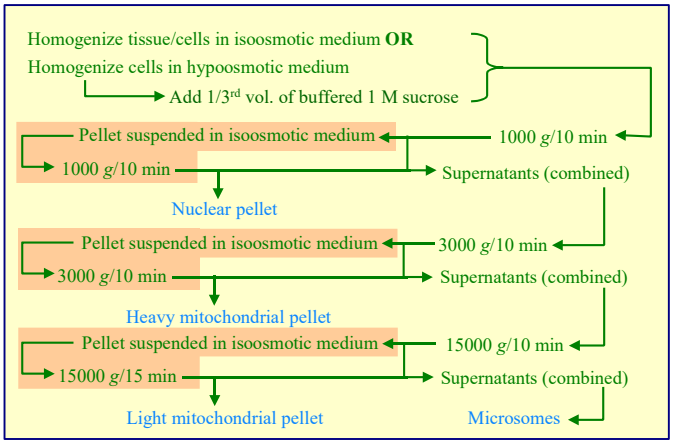


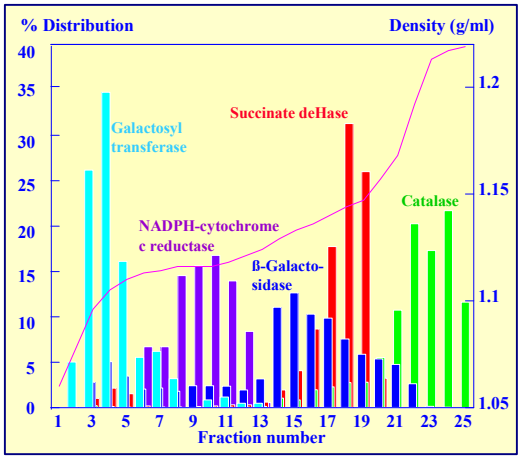
Figure 1: Differential centrifugation scheme for mammalian tissue and cell homogenates; for information see text

mitochondria, particularly those from cultured cells, which are more slowly sedimenting than those from a tissue such as liver. Sometimes the *g*-force used to produce the LMP is increased to 17,000 *g* [3,19,20]. The LMP will contain all of the remaining mitochondria, lysosomes and peroxisomes plus some of the more rapidly sedimenting microsomal vesicles, principally the rough endoplasmic reticulum (RER). Most of the microsomal vesicle populations will remain in the LMP supernatant, although any large plasma membrane fragments and/or tubular Golgi elements may be in the HMP or LMP. In any differential centrifugation scheme such as that shown in Figure 1, the organelles that sediment at a particular *g*-force often trap smaller particles in the process. This is often reduced by resuspending the pellet in the homogenization medium; repeating the centrifugation and combining the supernatants (see orange zones in Figure 1). The downside of this procedure is that the preparation time is increased and resuspension of the pellet may cause fragmentation of the organelles, unless the process is done very gently.

In gradient purification of mitochondria, the 3000 *g* sedimentation is frequently omitted and a total HMP+LMP fraction produced from the post-nuclear supernatant (PNS) [2,19-23]. To reduce contamination from microsomal vesicles the *g*-force used to pellet the total mitochondrial fraction from the PNS may be reduced to 10,000 *g* [8], 11,000 *g* [24] or 12,000 *g* [25-27].

There are also many examples in which the gradient input is the total PNS [1,9-13,28-33]. The advantages of this strategy are the considerable saving of time and the minimization of any loss of organelles; disadvantages are that the huge variety of particles in the sample severely tests the resolving power of the gradient and the possible inconvenient size of the sample. It may therefore be necessary to concentrate the particles by centrifugation at 100,000 *g* for 45-60 min [15,34,35]. If the study requires simultaneous analysis of mitochondria and other microsomal compartments such as the ER or Golgi, using a PNS may be the best option. Removal of the cytoplasmic proteins in the 100,000 *g* supernatant may also be an advantage. A problem with pelleting all the organelles at 100,000 *g*, aside from the considerable increase in preparation time and tendency of particles to aggregate together, might be the sensitivity of mitochondrial function to hydrostatic pressure.

- The differential centrifugation of brain homogenates has sometimes been omitted entirely and replaced by filtration of the homogenate through a 5 µm nylon mesh at 20 *g* for 10 min [7,36]. This may be particularly effective for removing nuclei and myelin fragments.



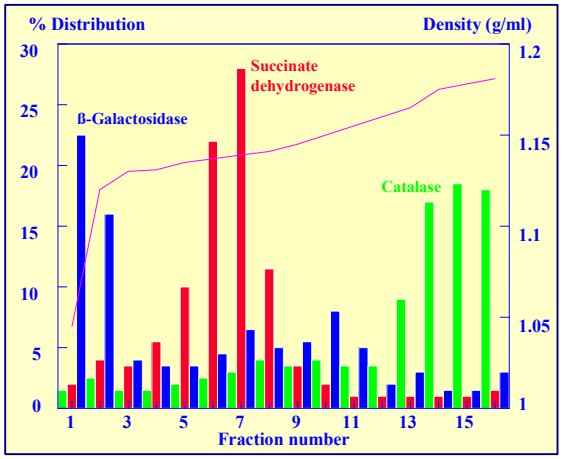


Figure 2 (left) Resolution of Golgi (galactosyl tranferase), ER (NADPH cytochrome c reductase), lysosomes (β-galactosidase), mitochondria (succinate dehydrogenase) and peroxisomes (catalase) of a mouse liver LMF by flotation through a 10-30% (w/v) iodixanol gradient. **Figure 3 (right)** Resolution of lysosomes, mitochondria and peroxisomes in a 19-27% (w/v) iodixanol gradient.

4. Gradient centrifugation

4a. Continuous gradients

The first report of the use of iodixanol [37] for the resolution of organelles from a crude mitochondrial fraction from mouse liver used a 10-30% (w/v) continuous gradient that was bottom loaded with the sample in 35% (w/v) iodixanol and centrifuged at 52,000 *g* for 1.5 h (Figure 2). The analysis showed the potential of iodixanol gradients since all of the membrane particles, Golgi (galactosyl transferase), ER (NADPH-cytochrome c reductase), β-galactosidase (lysosomes), succinate dehydrogenase (mitochondria) and catalase (peroxisomes) displayed a characteristic median density. Subsequently, a more shallow gradient of 19-27% iodixanol was used

to improve the resolution of the major organelles (Figure 3). Examples of the use of continuous gradients are given in Table 2.

Table 2 Continuous iodixanol gradients for the purification and analysis of mitochondria[1]

Tissue/cell type	Gradient (% iodixanol)	g/time(h)	Study topic	Ref #
Brain	HM 12-38	100,000/1.5	Amyotrophic lateral sclerosis	7,36
Carcinoma cells	HMP+LMP 19-27	70,000/2.0	Apoptopic effects of hsp90	8
	PNS 2.5-27.5	200,000/3.0	Mcl-1 targeting in apoptosis	30
	PNS 5-25	100,000/3.0	Oxidoreductin in onset of apoptosis	9
	HMPor LMP 10-30	80,000/2.0	Regulation of Coenzyme A synthase	38
	10-30 LMP	100,000/3.0	TARP in androgen regulation	39
	10-30 LMP	52,000/1.5	ARL2 and BART locate to mitochondria	6
	10-30 LMP	52,000/1.5	Alzheimer's molecules localization	40
	LMP+MIC 10-30	150,000/18	Prolyl-4-hydroxylase domain proteins	15
HEK cells	PNS 2.5-27.5	20,000/3.0	Mcl-1 targeting in apoptosis	29
	PNS 2.5-27.5	20,000/3.0	K^+_{ATP} channel Kir6.1 subunit location	31
Hepatocytes/hepatoma	10-30 PNS	52,000/1.5	Myristic acid effect on ceramide synthesis	32
	10-30 PNS	52,000/1.5	Connexin 43 localization	33
Kidney	HM 12-38	100,000/1.5	Amyotrophic lateral sclerosis	7
Liver	HM 12-38	100,000/1.5	Amyotrophic lateral sclerosis	7
Mammary epithelium	10-30 HMP+LMP	52,000/1.5	Lysosomal studies	41
Mouse embryo fibro.	LMP+MIC 2.5-30	200,000/3.0	Apoptosis linked to Bcl-2 reductions	28

[1] If the sample is layered on top of the gradient, HM (homogenate), PNS (post-nuclear supernatant), HMP (heavy mitochondrial pellet), LMP (light mitochondrial pellet) or MIC (microsomes) appear before the % iodixanol figures; if the sample is layered beneath the gradient, HMP etc. appears after the % iodixanol figures. In some cases the principal study may be an organelle other than mitochondria, the method nevertheless provides excellent resolution of mitochondria.

4b. Discontinuous gradients

A three-layered discontinuous flotation gradient of 1.079 g/ml (10% iodixanol), 1.175 g/ml (30% w/v iodixanol) and approx 1.2 g/ml (35-36% iodixanol), the latter containing the crude mitochondria, was first described by Zhou et al [27] for the purification of mouse liver mitochondria from both lighter and denser organelles (see Figure 4). Choi et al [24] used a similar strategy for HEK293 mitochondria but based the method on one for the purification of yeast mitochondria by Meeusen et al [42] in which the density of the three layers was 1.10 (14.5 % iodixanol), 1.16 (27% iodixanol) and 1.27 g/ml (50% iodixanol).

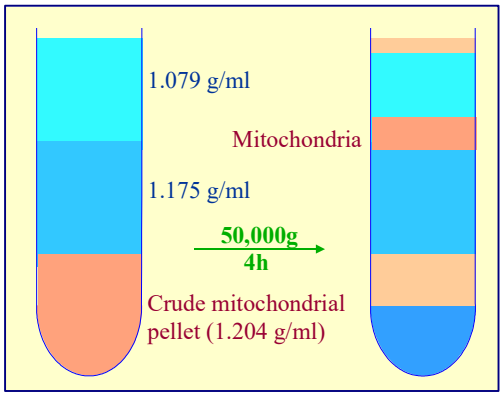


Figure 4: Purification of mitochondria in a discontinuous flotation gradient

Bottom-loaded flotation gradients can certainly provide excellent resolution of organelles; from a functional point of view however top-loading may be preferred because of the known sensitivity of mitochondria to hydrostatic pressure [43], which is highest at the bottom of the tube of a swinging-bucket rotor. In contrast to continuous gradients, discontinuous gradients offer the easy compromise of median-loading and this was first reported by Graham et al [44] in which the LMP in 25% Nycodenz®, was underlayered by 40% Nycodenz® and overlayered by 23%, 20%, 15% and 10% Nycodenz®. The gentle centrifugation conditions of 52,000 *g* for 1.5 h might also be well suited to the isolation of functionally competent mitochondria. While there was good separation of mitochondria from lysosomes and lysosomes from Golgi membranes in the 10-23% region of the gradient, resolution of mitochondria from peroxisomes was relatively poor. This was subsequently remedied by the introduction of additional layers of 30% and 34% Nycodenz® [45], which allowed the peroxisomes to band at the 34%/40% Nycodenz® interface. Teoh et al [46] identified the material at each interface by Western blotting of SDS-PAGE analyzed fractions. Material at the 25%/34% interface was rich in cytochrome oxidase but completely lacking in catalase (peroxisomes) and GRP78 (endoplasmic reticulum). The median-loaded strategy has been very successfully adapted to the use of iodixanol [47] for HEK cells and for rat liver [48].

The vast majority of published papers have reported the use of discontinuous gradients with the crude fraction either top- or bottom-loaded and some of these are given in Table 3. Table 3 also includes examples of the use of a hybrid gradient incorporating 6% (v/v) Percoll™ over 17% and 35% (w/v) iodixanol [1], which was introduced for neonatal cardiomyocytes. It may be beneficial for some cell types in which the peroxisomes and

mitochondria have rather overlapping densities. Percoll™ produces a pronounced shift in the density of peroxisomes to very low values.

- Note that in bottom-loaded formats any cytoplasmic proteins remain at in the sample zone.

Table 3 Discontinuous iodixanol gradients for the purification and analysis of mitochondria[1]

Tissue/cell type	Gradient (% iodixanol)[1]	*g*/time(h)	Study topic	Ref #
Brain	10,30,36(HMP+LMP)	50,000/4.0	Amyotrophic lateral sclerosis	2,19,21
Carcinoma cells	10,30,36(LMP)	50,000/4.0	P-glycoprotein	14
	10,30,36(LMP)	50,000/4.0	ATP-binding casette transporter (ABCB6)	15
	10,30,36(LMP)	50,000/4.0	Breast cancer resistance protein	18
Cardiomyocytes	(HMP+LMP),6P,17,35	50,000/0.5	Cytochrome c oxidase activity control	1
	(HMP+LMP),6P,17,35	50,000/0.5	F_1F_0 ATPase and F_1F_0 ATP synthase	11-13
Heart	(HMP+LMP),19,27	70,000/2.0	Regulation of mitochondrial DNA	4
	(HMP+LMP),19,27	70,000/2.0	Fatty acid biosynthesis	49
	(HMP+LMP),6P,17,35		PKC-ε interaction with cyt. ox. IV subunit	25,26
	10,30,36(LMP)	50,000/4.0	Mitochondrial dysfunction in hypertrophy	23
HEK cells	14.5,27,50(HMP+LMP)	80,000/3.0	Mitochondrial DNA	24
Leukaemia cells	10,30,36(LMP)	50,000/4.0	Oxidative stress-related proteins	22
Liver	10,30,36(HMP+LMP)	50,000/1.5	SOD following ischaemia/reperfusion	27
	10,30,36(HMP+LMP)	50,000/4.0	SOD1 (mutant form) mit. dysfucntion	2
Macrophages	10/17.5/25/35(HMP)	50,000/2.0	Cardiolipins and sterols	17
Mouse embryo fibro.	LMP,10,40	50,000/4.0	Fatty acid induced apoptosis	3
Spinal cord	0/20/25/44(HMP+LMP)	170,000/3.0	SOD1 in motor neuron injury	20

[1] If the sample is layered on top of the gradient, HMP (heavy mitochondrial pellet), LMP (light mitochondrial pellet) appear before the % iodixanol figures; if the sample is layered beneath the gradient, HMP etc. appears after the % iodixanol figures. 6P = 6%(v/v) Percoll™.

More discriminating multi-step discontinuous gradients may be able to give concomitant and significant enrichments of other membranes. For example a gradient of 10,15,20,25 and 30% (w/v) iodixanol on to which a concentrated liver PNS fraction was loaded and centrifuged at 100,000 *g* for 3 h provided an initial resolution of Golgi, ER and lysosomes; the mitochondria+peroxisome fraction was recovered from the bottom fractions of the gradient [34,35]. The mitochondria and peroxisomes were then completely resolved in a second gradient of 20, 25, 30 and 35% iodixanol run under the same centrifugation conditions.

4c. Self-generated gradients

The formation of self-generated gradients requires higher *g*-forces than might be used with a pre-formed gradient. But, because the sedimentation path length of the rotors used for self-generated gradients (vertical, near-vertical or some fixed-angle rotors) is much lower than that of a swinging-bucket rotor, the hydrostatic pressure on the organelles is rather similar in the two systems. Compared to pre-formed gradients, there are three advantages to the use of self-generated gradients for organelle fractionation. (1) Multiple samples can be handled far more easily. (2) Resolution in any pre-formed gradient may be affected by the high particle concentration in the sample and the build-up of particles at interfaces during centrifugation. With pre-formed gradients this can be avoided only by incorporating the sample into the whole gradient (not always very convenient) - but this ideal is a natural consequence for self-generated gradients as the sample is simply mixed with a suitable solution of iodixanol. (3) Gradient density profiles and hence fractionations are far more reproducible than with pre-formed gradients.

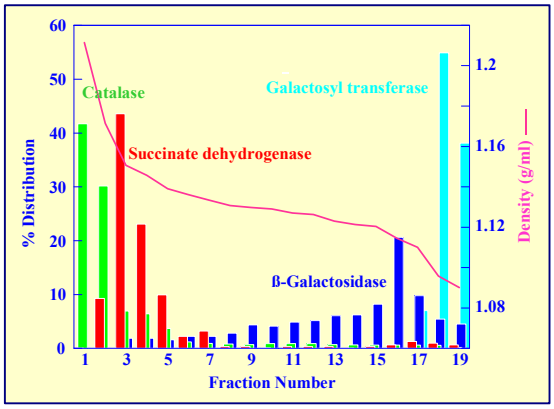


Figure 5: Separation of major organelles from a mouse liver LMP in a self-generated iodixanol (20%) gradient, centrifuged at 180,000g_{av}, for 3 h in a fixed-angle rotor (10 ml tube, 20° angle)

Figure 5 shows a typical separation from ref 37. The methodology is probably more useful for studying the localization of a molecule or function amongst different membranes; i.e. the gradient is used analytically rather than preparatively for the isolation of one specific organelle. In the literature however self-generated gradients have largely been used for studying lysosomal rather than mitochondrial function.

5. Technical information

Detailed methods for the purification of mitochondria are described in three OptiPrep™ Application Sheets (S12-S14); these can be accessed via the following website www.Optiprep.com. Click on "Methodology", then "Subcellular membranes" and follow the links from the index. Some ancillary OptiPrep™ Application Sheets on solution making, pre-gradient techniques, gradient preparation and gradient analysis are also listed below and may be accessed from the preface to the index.

- Application Sheet S14 describes the purification of mammalian mitochondria in a discontinuous iodixanol gradient; scroll down to "**Mitochondria**" in the index
- Application Sheet S15 describes the purification of mammalian mitochondria in a continuous iodixanol gradient; scroll down to "**Light mitochondrial fraction, analysis**" in the index
- Application Sheet S16 describes the purification of mammalian mitochondria in a self-generated iodixanol gradient; scroll down to "**Light mitochondrial fraction, analysis**" in the index
- Application Sheet S01 – Preparation of gradient solutions
- Application Sheet S03 – Preparation of continuous and discontinuous gradients
- Application Sheet S04 – Preparation of self-generated gradients
- Application Sheet S05 – Homogenization of mammalian tissues
- Application Sheet S06 – Homogenization of mammalian cells
- Application Sheet S07 – Differential centrifugation of homogenates
- Application Sheet S08 – Gradient harvesting
- Application Sheet S09 – Gradient analysis

6. References

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