# Fractionation of rough and smooth endoplasmic reticulum and subfractionation of ER from cultured mammalian cells

- OptiPrep™ is a 60% (w/v) solution of iodixanol in water, density = 1.32 g/ml
- **To access other Application Sheets** referred to in the text: return to the **2020SMemapp** file and select the appropriate S-number.
- An alternative self-generated iodixanol gradient protocol is described in **Application Sheet S18**
- See Section 3 for a comment on the use of pancreatic tissue.
- Section 4 contains some comments about ER subfractionation.
- An **OptiPrep™ Reference List (RS05)** "Analysis of membrane trafficking in mammalian tissues and cells: fractionation of ER, Golgi, TGN, PM and endosomes" provides a bibliography of all published papers reporting the use of OptiPrep™ for analysis of these membranes: to access return to the initial list of Folders and select "**Reference Lists**".
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## 1. Sedimentation velocity gradients

### 1a. Background

For the analysis of protein synthesis and translocation, the widely-used routine method for separation of the smooth and rough endoplasmic reticulum (SER and RER), which was devised by Walter and Blobel [1], involves a simple 1.3 M sucrose density barrier. In this system the SER bands at the interface while the RER and ribosomes pellet. Call et al [2] noted however that for some cell types the method did not yield high activity ER microsomes.

The protocol in this Application Sheet describes an alternative discontinuous iodixanol gradient that separates the SER and RER by sedimentation velocity. Although the gradient set-up time is longer than that of the self-generated gradient or the sucrose density barrier, the centrifugation time is only 30 min and uses a routine swinging-bucket rotor. As with the self-generated gradient, the RER forms a band in the gradient, rather than the gelatinous co-pellet with the ribosomes of the sucrose barrier technique, so its recovery from the gradient is rather easier. The method may also permit some subfractionation within both the SER and RER bands.

Although the banded material in the gradient is normally recovered by collection of the gradient in 10-20 equal volume fractions, the major SER and RER zones are so clearly defined that they may also be retrieved using a syringe.

The method was first described by Majoul et al [3] for analyzing KDEL protein transport from the plasma membrane to the ER in Vero cells and much more recently by Sannerud et al [4] in the analysis of ER-Golgi trafficking. The protocol is adapted from refs 3 and 4.

### 1b. Solutions required (see Section 1e, Note 1)

**A.** OptiPrep™
**B.** Phosphate buffered saline
**C.** Cell wash medium: 140 mM NaCl, 30 mM KCl, 10 mM EDTA, 25 mM Tris-HCl, pH 7.4
**D.** Homogenization medium: 130 mM KCl, 25 mM NaCl, 1 mM EGTA, 25 mM Tris-HCl, pH 7.4

Keep the following stock solution at 4°C:
1 M Tris (free base): 12.1 g per 100 ml water
1 M NaCl: 5.84 g per 100 ml water
1 M KCl: 7.45 g per 100 ml water
100 mM EDTA ($Na_2$•$2H_2O$): 3.72g per 100 ml water
100 mM EGTA (free acid): 3.80 g per 100 ml water (pH 11-12)

Solution C: Mix 28 ml, 6 ml, 20 ml and 5 ml, respectively of the NaCl, KCl, EDTA and Tris stock solutions; adjust to pH 7.4 with 1 M HCl and make up to 200 ml.

Solution D: Mix 5 ml, 26 ml, 2 ml and 5 ml, respectively of the NaCl, KCl, EGTA and Tris stock solutions; adjust to pH 7.4 with 1 M HCl and make up to 200 ml.

**1c. Ultracentrifuge rotor requirements**

Any swinging-bucket rotor, capable of approx 150,000g, with approx. 13-14 ml tubes (e.g. Beckman SW 41 or Sorvall TH641) or approx 17 ml tubes (e.g. Beckman SW28 or SW28.1 or Sorvall AH629)

**1d. Protocol**

Carry out all operations at 0-4°C

- Although the published method involved construction of a multi-step continuous gradient, the gradient becomes essentially continuous and linear during preparation and centrifugation. A more simple option may be to make a continuous gradient initially (see options in Steps 1 and 5)

1. For the discontinuous gradient: prepare solutions of 5%, 7.5%, 10%, 12.5%, 15%, 17.5%, 20%, 22.5% and 25% (w/v) iodixanol by diluting OptiPrep™ with Solution D **OR** for the continuous gradient prepare 5% and 25% (w/v) iodixanol only (**see Section 1e, Note 2**).

2. Wash the cells twice in Solution B and twice in Solution C.

3. Resuspend the cells in Solution D and prepare an homogenate using a ball-bearing homogenizer (cell cracker) or by repeated passage (approx. 10 passages) through a narrow gauge syringe needle (24-25G) or use a tight-fitting Dounce homogenizer (**see Section 1e, Notes 3 and 4**)

4. Remove the nuclei and heavy mitochondria fractions by centrifugation at 3000 *g* for 10 min (**see Step 5 and Section 1e, Note 5**).

5. During the centrifugation prepare a discontinuous gradient from 1.2 ml (13 ml tube) or 1.7 ml (17 ml tube) each of the nine iodixanol solutions **OR** using a two-chamber gradient maker or Gradient Master™ from equal volumes (5.5 ml or 7.5 ml) of the two iodixanol solutions for the 13 or 17 ml tubes respectively (**see Section 1e, Note 6**).

6. Layer 0.5-1.0 ml of the supernatant from step 4 on top of each gradient (see Section 1e, Note 7).

7. Centrifuge at 126,000 *g* for 30 min.

8. Harvest the gradients by upward displacement with a dense solution, by tube puncture or aspiration from the meniscus in 0.5-1.0 ml fractions for analysis (**see Section 1e, Notes 8 and 9**).

**1e. Notes**

1. Protease inhibitors (PMSF, leupeptin, antipain, aprotinin etc) may be included in any or all of the media at the operator's discretion. Strategies for preparing working solutions for mammalian tissues are given in **Application Sheet S01**.
2. Majoul et al [3] used a slightly different discontinuous iodixanol gradient in which the two densest solutions were 25% and 30% (w/v) iodixanol rather than 22.5% and 25%.
3. Some cells may require osmotic swelling prior to homogenization. The hypoosmotic medium can be a simple low concentration buffer such as 10 mM Hepes-KOH, pH 7.5. Always return the homogenate to isoosmotic conditions as soon as possible. For more information about homogenization of cells **see Application Sheet S06**.
4. Note that the volume of the homogenate should be kept to a minimum; as the separation is based on sedimentation velocity only 0.5-1.0 ml of material may be placed atop each gradient.
5. An optional 1000 *g*/10 min step may be inserted prior to the 3000 *g* centrifugation. This may prevent large amounts of rapidly sedimenting nuclei and debris trapping a lot of other smaller particles. Note that in buoyant density fractionations (**see Section 2a**) it is common to use a high *g*-force (12,000 *g*) to remove the larger organelles.
6. For more information on construction of both discontinuous and continuous gradients **see Application Sheet S03**.

7. If the volume of the 3000*g* supernatant is inconveniently large, it may be necessary to pellet all of the particulate material at 100,000 *g* for 45 min and resuspending the pellet in a smaller volume. Centrifugation conditions for pelleting microsomes vary, Higashi et al [5] used 138,000 *g* for 1 h
8. For more information on the harvesting of gradients **see Application Sheet S08**.
9. Once the position of the SER and RER bands has been established it may be possible to remove them with a syringe. The SER peaks approx 1/3rd and the RER approx 2/3rd of the way down the tube.

**2. Buoyant density fractionations**

**2a. Discontinuous gradients**

In an alternative method devised by Kleene et al [6] for neuroblastoma cells, a microsomal fraction was sedimented from a 12,000 *g*/15 min supernatant; this was then resuspended in 0.32 M sucrose, 10 mM Tris-HCl, pH 7.4 and adjusted to 20% (w/v) iodixanol. It was layered between 30% and 15% iodixanol and centrifuged at 150,000 *g* for 3 h. The SER banded at the 15%/20% iodixanol interface and the RER 20%/30% iodixanol interface. This is a good example of using flotation of one membrane type and sedimentation of another to maximize resolution.

The strategy of layering the crude microsomes in 20% (w/v) iodixanol between 30% and 15% iodixanol is part of that the protocol recommended in the Sigma-Aldrich Endoplasmic Reticulum Isolation Kit. Its use has been reported in publications for neuroblastoma cells [7,8] and mouse pancreas [9]. Some methods omit the light mitochondrial centrifugation step and centrifuge a post-nuclear supernatant (PNS) at 200,000 *g* [9]; the presence of all of the other major organelles (mitochondria, peroxisomes and lysosomes) in the gradient input will however severely test the gradient's efficacy. The inclusion of a 12,000 *g* centrifugation of the PNS is strongly recommended.

More recently Ramming et al [10] used a discontinuous gradient of 20%, 16.25%, 12.5%, 8.75% and 5% (w/v) iodixanol, centrifuged at approx. 100,000 *g* for 3 h in a small volume swinging-bucket rotor (2.2 ml tubes).

**2b. Continuous gradient**s

Geiger et al [11] sedimented (100,000 *g* for 1 h) all of the membranes from a post-nuclear supernatant from CHO cells; resuspended them in 0.25 M sucrose, 1 mM EDTA, 10 mM Hepes-NaOH Tris-HCl, pH 7.4 and layered 0.5 ml on top of an approx. 13 ml 0-15% (w/v) iodixanol gradient (prepared by dilution of OptiPrep™ with the same medium). After centrifugation at 75,000 *g* for 18 h, the RER banded at the bottom of the gradient and the SER→ERGIC banded at the top. A rather broader density range (0-26% iodixanol) was used by Uribe et al [12] for macrophages. A 2000 *g* supernatant from a HeLa cell homogenate was adjusted to 40% (w/v) iodixanol and loaded beneath a 0-35% (w/v) iodixanol gradient. Centrifugation at 94,000 *g* for 17 h separated a broadly banded ER from the cytoskeleton, which remained at the bottom of the gradient [13]. Bottom-loading is certainly the sample-loading method of choice in resolving potentially dense protein components from membranes.

**3. Analysis of pancreatic RER**

Hori et al [14] incubated RER (isolated by differential centrifugation) in either a low- or high-salt buffer and then layered them on to a 20-30% (w/v) iodixanol gradient and centrifuged in a small volume fixed-angle rotor (Beckman TLA100.4) and centrifuged at approx. 100,000 $g_{av}$ for 140 min.

**4. Subfractionation of ER**

There is evidence that iodixanol gradients are able to fractionate ER on the basis of its function and cytoplasmic localization; Woods et al [15] were able to use a continuous iodixanol gradient to separate perinuclear ER from 3T3 cells on the basis of its paxillin enrichment. **For more details see Application Sheet S22**. Separation of transitional and peripheral ER from mouse embryo fibroblasts was achieved on 10-40% (w/v) iodixanol gradients centrifuged at 48,000 *g* for 18 h [16]. **See also Application Sheet S41**.

More recently use of a 10-34% (w/v) iodixanol gradient (approx 100,000 *g* for 16 h) showed that α1,2 mannosidase I located to a distinct subset of the ER; its sharp-peaked distribution pattern contrasted markedly with a broad distribution of a general ER-marker (calnexin) and was quite distinct from the Golgi [17,18]. A high-density fraction of liver tissue ER harvested from a sucrose gradient has also been further fractionated in an iodixanol gradient in a study of non-alcoholic steatohepatitis [19]. Wang et al [20] showed that the ER distribution of unesterified cholesterol in a 6-27% iodixanol gradient was altered by reductions in intracellular $Ca^{2+}$ of mouse embryonic fibroblasts.

## 5. References


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