University of Teramo

Course of Study in Biotechnology - **Biochemical Methodologies – 2nd Year**

PRACTICAL SESSION 3 – Isopycnic Centrifugation for Mitochondrial Isolation Using a Sucrose Density Gradient

Date: 13/11/2026

In this practical session, you will use **isopycnic centrifugation** — i.e., density gradient centrifugation — to isolate mitochondria from different biological starting materials: half of the groups will work with mouse liver tissue, the other half will start from a cell pellet – randomly distributed in the ice boxes. Both groups will then follow the same differential centrifugation and density gradient procedure.

In Practical Session 1, you already prepared TE buffer (10 mM Tris-HCl pH 7.4 + 1 mM EDTA). In this session, the first step is to prepare the STE isolation medium by adding sucrose to 50mL of TE buffer. The entire procedure is carried out at **0–4 °C**: always keep samples on ice.

Theoretical Background

✎ **Question** What is the difference between differential centrifugation and isopycnic (density gradient) centrifugation? In both cases, which physical property of the particle determines its separation?

✎ **Question** Why must the isolation medium contain sucrose?

✎ **Question** Mitochondria have an equilibrium density of approximately 1.19–1.21 g/mL. Looking at the gradient you will use (layers at 15%, 23%, 31%, 40%, and 55% sucrose, with densities of 1.057 – 1.093 – 1.128 – 1.172 – 1.238 g/mL, respectively), at which interface do you expect to find the mitochondrial band? Justify your answer.

Materials and Equipment

Solutions (kept on ice):

- TE buffer prepared in Practical Session 2 (10 mM Tris-HCl pH 7.4 + 1 mM EDTA) – aliquoted in 50mL falcon tubes.
- Pure sucrose (MW = 342.30 g/mol) — to be weighed at the bench for STE medium preparation
- Gradient solutions (prepared in the 1st lab experience): 15%, 23%, 31%, 40%, 55% sucrose in TE buffer

Samples:

- **Group A:** mouse liver tissue
- **Group B:** cell pellet

Equipment:

- Technical balance
- Ice
- Pestle
- Glass Pasteur pipette
- Beaker
- 15 mL Falcon tubes, 1.5 mL Eppendorf tubes
- Micropipettes and tips
- Refrigerated bench-top centrifuge with swing-out rotor
- Refrigerated microcentrifuge (≥ 10,000 × g)

1. Preparation of the STE Isolation Medium

The STE medium (Sucrose + Tris + EDTA + BSA) is the buffer used for homogenisation and all mitochondrial pellet washes. It is prepared by adding sucrose and BSA to the TE buffer already available in the laboratory.

Composition of STE medium

250 mM Sucrose (MW: 342.30 g/mol): provides osmotic support (≈ 290 mOsm) and prevents mitochondria from swelling and lysing.

TE BUFFER (already aliquoted in 50mL falcon tubes on the benches) composed by: 10 mM Tris-HCl pH 7.4 + 1 mM EDTA + BSA 0.1% (w/v) -Tris buffers for acidification of the homogenate, EDTA chelates divalent metals and inhibits calcium-dependent proteases; BSA binds free fatty acids released from damaged membranes, which would otherwise uncouple the mitochondrial respiratory chain.

FOR 50 mL OF STE ISOLATION MEDIUM:

Calculation of sucrose mass to be weighed

m (g) = _____

- Weigh the calculated amount of sucrose on the technical balance: _____ g. Record the actual weight.
- Add the sucrose to the 50 mL of TE buffer in the Falcon tube and mix by gentle end-over-end inversion until completely dissolved.

2. Sample Preparation

Homogenisation of Liver Tissue and Cell Pellet

PURPOSE: to mechanically disrupt cell membranes in order to release organelles without damaging them. Work on ice, using slow movements to avoid producing foam.

- Add 1 mL of STE medium and 10 µL of protease inhibitor (F_d 100X) to the sample
- Homogenise with a pestle
- Pipette to mix
- Transfer the homogenised sample into a 15 mL Falcon tube, then add 4 mL of STE medium and 40 µL of protease inhibitor.

3. Differential Centrifugation

Before loading onto the gradient, it is necessary to enrich the mitochondrial fraction and remove nuclei, cell debris, and cytosol by differential centrifugation.

Table 1. Centrifugation steps (all at 4 °C).

Step	RCF × time
C1 –clarification step	750 × g, 5 min
C2 – Mitochondria collection	10,000 × g, 6 min

- Perform the first centrifugation at 750 × g, 5 min, 4 °C.
- Collect 1 mL of supernatant and transfer it to a 1.5 mL Eppendorf tube.
- Perform centrifugation at 10,000 × g, 6 min.
- Discard the supernatant and resuspend the pellet in 1 mL of cold STE.

4. Discontinuous Sucrose Gradient (15–55%)

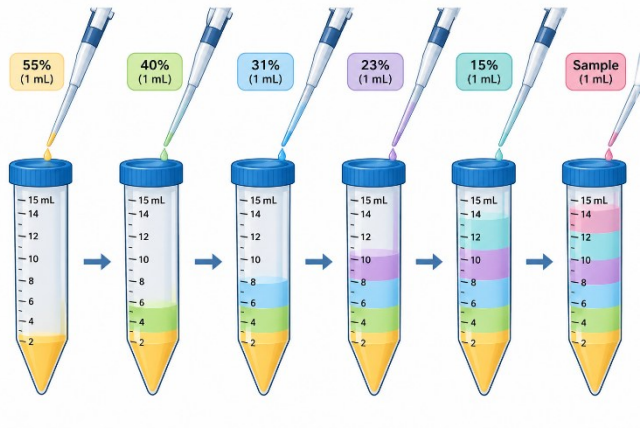
The gradient is assembled in a 15 mL Falcon tube by layering **1 mL of each of the five fractions**, starting from the densest (bottom) to the least dense (top), and loading 1 mL of sample on top: total volume 11 mL (see the attached layering diagram).

The three fundamental rules for this run

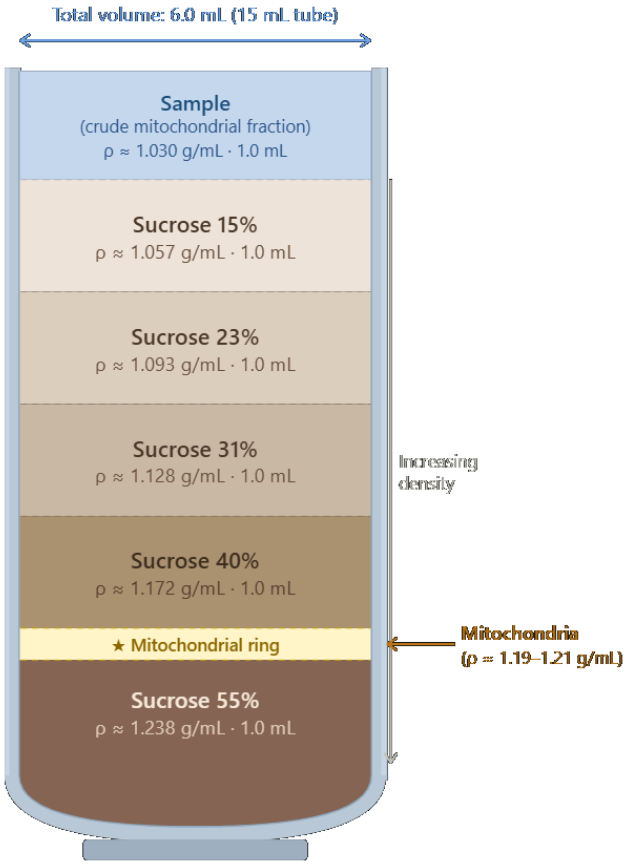
1. Always layer from bottom to top, starting with the densest solution: the 55% goes at the bottom, the 15% at the top. A denser liquid remains spontaneously below a less dense one only if it is not disturbed during loading.

2. The sample (1 mL of STE with crude mitochondria) is deposited on top of the 15% layer. Its density (STE ≈ 1.030 g/mL) is lower than that of the 15% layer (ρ ≈ 1.057 g/mL), so it remains at the surface until centrifugation begins.

3. The brake must be DISABLED and acceleration set to the slowest ramp. Abrupt deceleration remixes the interfaces and destroys the separation: this is the most common error in a gradient run.



- Deposit 1 mL of 55% sucrose (ρ ≈ 1.238 g/mL) at the bottom of the tube; this layer acts as a cushion for heavier material.
- Layer on top, in order: 1 mL of 40% (ρ ≈ 1.172 g/mL), then 1 mL of 31% (ρ ≈ 1.128 g/mL), then 1 mL of 23% (ρ ≈ 1.093 g/mL), and finally 1 mL of 15% (ρ ≈ 1.057 g/mL).
- With maximum care, load 1 mL of crude mitochondrial fraction on top of the 15% layer, allowing it to run down along the wall of the tube.
- Position the Falcon tubes at diametrically opposite positions in the rotor.
- Centrifuge at 5,000 × g for 15 minutes.
- At the end, wait for the rotor to come to a **complete stop** before opening. Remove the tubes without shaking them. Observe them against the light.



Where is the mitochondrial band?

Mitochondria (ρ ≈ 1.19–1.21 g/mL) collect at the interface between the 40% and 55% layers: the **second-to-last and last band** counting from the top. This ring appears as a whitish, milky, and slightly opaque band. It is the most enriched band.

- Using a glass Pasteur pipette, reach the layer corresponding to 55%, collect it, and transfer it to an Eppendorf tube. The resulting sample will subsequently be used for mitochondrial protein quantification.