Product Information

Endoplasmic Reticulum Isolation Kit

For isolation of intact ER from mammalian soft tissues and cultured cells

ER0100

Product Description

Storage Temperature: 2-8 °C

The endoplasmic reticulum (ER) is a network of interconnected tubules, vesicles, and sacs. The ER plays a role in specialized cellular functions, including:

- protein synthesis
- sequestering of calcium
- production of steroids
- storage and production of glycogen
- insertion of membrane proteins

The Endoplasmic Reticulum Isolation Kit provides a method for isolating the ER from animal soft tissues (liver, spleen, and kidney) and from cultured cells by differential centrifugation.[1-3] The ER0100 kit contains all the reagents required for ER preparations with various degrees of purity:

- crude ER (microsomes)[3]
- Ca^{2+}-precipitated rough ER (RER) enriched microsomes[4,5]
- density gradient-purified rough ER (RER) and smooth ER (SER)[6]

The ER isolation process can be monitored by measuring NADPH cytochrome c reductase activity (such as with the Cytochrome c Reductase Assay Kit, Cat. No. CY0100). This enzyme is an ER membrane protein that is commonly used as an ER marker.[4]

The isolated ER can be used to study the cytochrome P450 system and xenobiotic metabolism, to study lipid metabolism, and to recover ER membrane and lumenal proteins. ER0100 has been tested with:

- rat liver, kidney, and brain
- mouse liver
- rabbit liver, kidney, spleen, heart, and brain
- Jurkat and HeLa cell lines

Several dissertations have cited use of ER0100 in their protocols.[9-18]

Precautions and Disclaimer

This product is for R&D use only. Not for drug, household, or other uses. Please consult the Safety Data Sheet for information regarding hazards and safe handling practices.

Components

- Isotonic Extraction Buffer 5×, 100 mL (Component I3533): 50 mM HEPES (pH 7.8), with 1.25 M sucrose, 5 mM EGTA, and 125 mM KCl
- Hypotonic Extraction Buffer 10×, 10 mL (Component H8412): 100 mM HEPES (pH 7.8), with 10 mM EGTA and 250 mM KCl
- Calcium Chloride Solution, 5 mL (Component C2052): 2.5 M $CaCl_2$ solution
- OptiPrep™ Density Gradient Medium, 100 mL (Component O3028): 60% (w/v) solution of iodixanol in water
- Needle, 4 inch, 20 gauge, 1 each (Component N4161)

Reagents and Equipment Required

(Not provided)

Example Cat. Nos. are given where appropriate.

- Protease Inhibitor Cocktail for use with mammalian cell and tissue culture extracts (such as Cat. Nos. P8340, PIC0002)
- Dulbecco's Phosphate Buffered Saline (DPBS, such as Cat. No. D8537)
- Ultrapure water
- Centrifuge with SS-34 head or equivalent
- High-speed centrifuge or ultracentrifuge, with fixed-angle head

- Appropriate homogenizer:
  - Potter-Elvehjem PTFE pestle in glass tube homogenizer:
    - 3 mL (Cat. No. P7734)
    - 8 mL (Cat. No. P7859)
    - 45 mL (Cat. No. P7984)
  - Pellet pestle
  - Motor for pellet pestle (Cat. No. Z359971)
  - Dounce glass tissue grinder set, 7 mL (Cat. No. D9063)
  - Overhead electric motor
- Scalpel, forceps, and glass plate
- Microcentrifuge tubes
- Pasteur pipettes
- Syringe, suitable for gradient separation (1 mL syringe for an 8 mL ultracentrifuge tube)

Storage/Stability

Store the unopened kit at 2-8 °C. When stored unopened, the components in this kit are stable for 24 months.

After opening the kit, the needle (Component N4161) and the OptiPrep™ Density Gradient Medium (Component O3028) can be stored at room temperature.

Procedure

Endoplasmic reticulum (microsomes) with different degrees of purity can be easily prepared from animal tissues or cultured cells using a simple method of homogenization with the aid of a PTFE pestle in a glass tube homogenizer, followed by differential centrifugation. Tissue culture cells may have to be preswollen in the 1× Hypotonic Extraction Buffer, before homogenization and differential centrifugation.

The serial centrifugations include:

- Low speed centrifugation (1,000 × g)
- Medium speed centrifugation (12,000 × g)

The serial centrifugations remove nuclei, cell debris, mitochondria, and lipids to give a post-mitochondrial fraction (PMF). This PMF 12,000 × g supernatant is the starting material for the preparation of ER microsomes.

From the PMF 12,000 × g supernatant, there are two options for microsomal preparation:

Option 1: Calcium chloride precipitation of the PMF supernatant, followed by a medium-speed centrifugation (8,000 × g), to obtain RER-enriched microsomes.

Option 2: Centrifugation of the PMF supernatant at 100,000 × g, to obtain a crude microsomal preparation. This preparation can be either used (a) as is, or (b) further separated into RER and SER, utilizing a self-generating density gradient medium (OptiPrep™) by ultracentrifugation.

A flow diagram for the various preparations of ER is shown in Appendix I.

Reagent Preparation

Note: Use ultrapure water for the reagent preparation.

1× Isotonic Extraction Buffer: 10 mM HEPES (pH 7.8), 250 mM sucrose, 25 mM KCl, and 1 mM EGTA

1. Aseptically remove an aliquot of the Isotonic Extraction Buffer 5× (Component I3533).
2. Dilute the aliquot 5-fold with water.
3. Keep the 1× Isotonic Extraction Buffer at 4 °C before use.
4. Just before use, add Protease Inhibitor Cocktail for mammalian cells (Component P8340) to the 1× Isotonic Extraction Buffer at a concentration of 1% (v/v).

Note: Suggested volumes of 1× Isotonic Extraction Buffer are as follows.

For **Option 1**:

- Use a minimal tissue weight of 0.5-1 g.
- Prepare 10 mL of buffer for 0.5-1 g of tissue weight.

For **Option 2 (a)**, **without gradient separation**:

- Use a minimal tissue weight of 2 g.
- Prepare 20 mL of buffer for 2 g of tissue weight.

For **Option 2 (b)**, **with gradient separation**:

- Use a minimal tissue weight of 4 g.
- Prepare 60 mL of buffer:
  - 40 mL for crude microsome preparation
  - 20 mL for the density gradient separation (when using two 8 mL centrifuge tubes).

1× Hypotonic Extraction Buffer: 10 mM HEPES (pH 7.8), 25 mM KCl and 1 mM EGTA (required for preparation of ER from cultured cells)

1. Dilute an aliquot of the Hypotonic Extraction Buffer 10× (Component H8412) 10-fold with water.
2. Keep the 1× Hypotonic Extraction Buffer at 4 °C before use.
3. Just before use, add the Protease Inhibitor Cocktail for mammalian cells (Component P8340) to the 1× Hypotonic Extraction Buffer at a concentration of 1% (v/v).
4. Prepare 3 mL of 1× Hypotonic Extraction Buffer for each mL of packed cells.

8 mM Calcium Chloride Solution:

1. Dilute an aliquot of the 2.5 M $CaCl_2$ Solution (Component C2052) with water, 0.032 mL of the 2.5 M $CaCl_2$ Solution per 10 mL of water.
2. Keep the 8 mM Calcium Chloride Solution at 4 °C before use.
3. Prepare 7.5 mL of the 8 mM Calcium Chloride Solution for each mL of PMF.

OptiPrep™ Density Gradient

For separation in 8 mL centrifuge tubes, prepare either:

- 10 mL of 30% OptiPrep™:
  - Dilute the OptiPrep™ Density Gradient Medium (60% w/v) **2-fold** with 1× Isotonic Extraction Buffer. Mix well.
- 10 mL of 15% OptiPrep™:
  - Dilute the OptiPrep™ Density Gradient Medium (60% w/v) **4-fold** with 1× Isotonic Extraction Buffer. Mix well.

Preparation of ER from animal tissues

- Perform the whole procedure at 4 °C. All the solutions and equipment should be pre-cooled before use.
- For samples < 1 g, use an 8 mL PTFE pestle in a glass tube homogenizer.
- For samples > 1 g, use a 45 mL PTFE pestle in a glass tube homogenizer.

1. Use a fresh tissue sample from an animal that was starved overnight and sacrificed the next morning.
2. Wash the tissue sample twice with 10 mL of PBS by placing in a dish and shaking gently for few minutes.

   Note: Step 2 is performed to remove blood from certain tissues, such as liver.

   2.1. Place the tissue on a paper towel to absorb excess liquid and blood clots, if present.

   2.2. Cut the tissue into small pieces (1.5-2 cm).

   2.3. Repeat the wash step.
3. Blot the tissue on a paper towel. Weigh.
4. Cut the tissue, with the aid of a scalpel and glass plate, into small slices (0.3-0.5 cm).

   4.1. Transfer the slices into a suitable glass homogenizer.

   4.2. Add 3.5 mL of the 1× Isotonic Extraction Buffer per gram of tissue.

   4.3. Homogenize the sample by an overhead motor (~200 rpm).

   4.4. Ensure total homogenization of the sample by moving the pestle up and down at least 7 times.

   4.5. Transfer the homogenate to a centrifuge tube.
5. Wash the PTFE pestle and glass vessel with 0.5 mL of the 1× Isotonic Extraction Buffer per gram of tissue.

   5.1. Add to the previous homogenate.

   5.2. Keep the homogenate on ice.
6. Centrifuge the homogenate at 1,000 × g for 10 minutes at 4 °C.

   6.1. Carefully remove the thin floating lipid layer by aspiration, being careful not to aspirate the post-nuclear supernatant.

   6.2. Transfer the supernatant to another centrifuge tube using a pipette.

   6.3. Discard the pellet.
7. Centrifuge at 12,000 × g for 15 minutes at 4 °C.

   7.1. Carefully remove the thin floating lipid layer by aspiration, being careful not to aspirate the post mitochondrial supernatant.

   7.2. Transfer the supernatant to another tube using a pipette. Discard the pellet.

   7.3. This supernatant fraction, which is the post-mitochondrial fraction (PMF), is the source for microsomes.

Note: For further analyses, it is recommended to save a sample (100-400 µL) before continuing with the next purification step.

Option 1: For isolation of RER-enriched microsomes (precipitation with $CaCl_2$)

Perform the following procedure at 4 °C.

1. Measure the volume of the PMF (V mL).
2. Prepare a volume of 8 mM Calcium Chloride Solution equivalent to 7.5× the volume of the PMF (V mL) (instructions in the Reagent Preparation section).
3. Transfer the PMF to a beaker (a size of 10× the volume of the PMF) that contains a suitable magnetic spinbar.
4. Add a volume of 8 mM Calcium Chloride Solution equivalent to 7.5× the volume of the PMF, dropwise, to the PMF with constant stirring. (A burette, Pasteur pipette, or separatory funnel may be used for dropwise addition of the solution.) The final concentration of $CaCl_2$ is 7 mM.
5. After all the 8 mM Calcium Chloride Solution is mixed with the PMF, stir for an additional 15 minutes at 4 °C.
6. Centrifuge the sample at 8,000 × g for 10 minutes at 4 °C. The enriched RER microsomes will be in the pellet.
7. Remove the supernatant. Suspend the pellet in 1× Isotonic Extraction Buffer (0.3 mL of buffer for each g of original tissue).
8. Place the suspension in a homogenizer.
   - If the volume of the suspension is small (< 0.8 mL), use a pellet pestle in a microcentrifuge tube.
   - If the volume is > 0.8 mL, use a 3 mL PTFE pestle in a glass tube homogenizer.
9. Homogenize completely by moving the pestle up and down several times at ~200 rpm.

Option 2: For isolation of the crude microsomal fraction (ultracentrifugation)

Perform the following procedure at 4 °C.

1. Centrifuge the PMF for 60 minutes at 100,000 × g in an ultracentrifuge at 4 °C. The PMF may be divided into two or more tubes. The pellet is the microsomal fraction.
2. Remove the supernatant using a pipette. Discard the supernatant.
3. The pellet is difficult to suspend. Therefore, transfer the pellet to an appropriate homogenizer with the aid of a spatula.
4. Wash each ultracentrifuge tube with 1× Isotonic Extraction Buffer (0.3 mL for each g of original tissue). Transfer the liquid to the homogenizer vessel.
   - If the volume of the suspension is small (< 0.8 mL), use a pellet pestle in a microcentrifuge tube.
   - If the volume is > 0.8 mL, use a 3 mL PTFE pestle in glass tube homogenizer.
5. Homogenize completely by moving the pestle up and down several times at ~200 rpm.

Notes: In some tissues (such as brain), the ER does not pellet well, and only 25% of the ER will be in this pellet. Liver tissue, on the other hand, will give 70-90 % of the ER in the pellet.

For further analyses, it is recommended to save a sample (100-400 µL) before continuing with the next purification step.

Self-Generating Density Gradient (OptiPrep™)

This procedure is for further purification and separation of RER (rough endoplasmic reticulum) and SER (smooth endoplasmic reticulum) from microsomes isolated by ultracentrifugation.

This procedure makes use of the self-generating density gradient medium iodixanol (OptiPrep™). Iodixanol is a low osmolarity, iodinated density gradient medium that is biologically inert and does not interfere with assays for marker enzymes. The crude microsomal fraction is adjusted to 20% (w/v) OptiPrep™ and is layered between the 30% and 15% OptiPrep™ layers. Following ultracentrifugation using a fixed angle rotor, fractions are separated from the top to the bottom of the gradient.

This procedure is suitable for 1-2 mL of the crude microsomal suspension (100,000 × g pellet), equivalent to 3.5-7 g of tissue or approximately 1-5 × 10^9 cells, when centrifuged in 8 mL ultracentrifuge tubes. For larger amounts, adjust the procedure accordingly.

Prepare a calibration scale sheet that suits the ultracentrifuge tubes used, to facilitate sample removal:

1. Affix a plain piece of paper, or preferably a small transparent sheet of plastic, to the outside of the ultracentrifuge tube to be used.
2. Add aliquots of water equal to the volume of the desired fractions (0.5 mL) to the tube. Mark the sheet with a fine-tipped marker at the height of the liquid. Use this as a guide for removal of identical aliquots after centrifugation.

Perform the following procedure at 4 °C.

1. Dilute the crude microsomal sample ("Preparation of ER from animal tissues", Option 2, Step 5) with the 60% OptiPrep™ Density Gradient Medium to a final concentration of 20% OptiPrep™:
   - Add 0.5 mL of the 60% OptiPrep™ Density Gradient Medium per 1 mL of sample. Mix well.
2. Place 2 mL of the 30% OptiPrep™ solution at the bottom of an 8 mL ultracentrifuge tube.
3. Carefully layer the sample containing 20% OptiPrep™ (Step 1), up to 1.5 mL, on top of the 30% layer with the aid of a Pasteur pipette, by placing drops on the wall of the tube close to the bottom layer. Ensure that the entire sample floats on top of the 30% OptiPrep™ solution cushion.
4. Layer 4 mL of the 15% OptiPrep™ solution very carefully on top of the sample with the aid of a Pasteur pipette, as described in Step 3.
5. Close the tube. Ensure that the balance tube is at the same weight as the sample tube. (A similar gradient or another sample may be used as balance.)
6. Centrifuge in an ultracentrifuge for 3 hours at 150,000 × g.
7. At the end of the run, carefully remove the tube. Clamp the tube to a stable base.
8. Affix the calibration scale sheet to the side of the tube with tape such that the upper meniscus coincides with a calibration line.
9. For an 8 mL centrifugation tube, withdraw fractions of 0.5 mL from the top of the gradient downwards using the supplied 4-inch blunt ended needle and a suitably sized syringe.
   9.1. Insert the end of the needle to the bottom of the first calibration line. Withdraw the aliquot.
   9.2. Record the volume withdrawn, as there may be variations between samples.
   9.3. Transfer the fraction to a microcentrifuge tube.
   9.4. Close the tube.
10. Continue to withdraw fractions in the same fashion by moving the needle to the bottom of the next calibration line.
    10.1. Transfer each fraction to a new microcentrifuge tube.
    10.2. Close the tube.
11. After removing the supernatant, wash the tube with 200 µL of 1× Isotonic Extraction Buffer to suspend any pellet.
12. Label the tubes containing the samples and perform appropriate assays. It may be useful to determine the following parameters:
    - Protein concentration with Bradford Reagent (10-fold dilution, 10-30 µL sample per test)[7]
    - NADPH cytochrome c reductase activity (ER marker): use 5-20 µL sample per test. Use of the Cytochrome c Reductase (NADPH) Assay Kit (Cat. No. CY0100) is suggested.
    - RNA detection[4,8] (10-50 µL sample per test) for the identification of RER, to which ribosomes are attached, in contrast to SER (which should be devoid of RNA)

Preparation of microsomes from cultured cells

Microsomes may also be prepared from tissue culture cells using the above procedure, except for the initial extraction procedure ("Preparation of ER from animal tissues" section, Steps 1-7), which may have to be altered as follows:

A. For adherent cells (such as HeLa cells, > 2 × 10^8 cells):

1. Detach the cells using conventional tissue culture methods.
2. Centrifuge the cells at 600 × g for 5 minutes. Remove the supernatant by aspiration.
3. Wash the cells with 10 volumes of PBS. Centrifuge as in Step 2.
4. Measure the packed cell volume (PCV).
5. Suspend the cells in a volume of 1× Hypotonic Extraction Buffer equivalent to 3 × PCV. Incubate the cells for 20 minutes at 4 °C to allow the cells to swell.
6. Centrifuge the cells at 600 × g for 5 minutes and remove the supernatant by aspiration. Measure the "new" PCV.
7. Add a volume of 1× Isotonic Extraction Buffer equivalent to 2× the "new" PCV. Transfer to a 7 mL Dounce homogenizer.
8. Break the cells with 10 strokes of the Dounce homogenizer. Then proceed to the differential centrifugation steps ("Preparation of ER from animal tissues" section, Steps 6-7).

B. For fragile cells (with "weak" outer membranes such as Jurkat cells):

1. Perform Steps 2-4 of the procedure for adherent cells above (Section A).
2. Suspend the cells in a volume of 1× Isotonic Extraction Buffer equivalent to 4 × PCV.
3. Homogenize the cells using a Dounce homogenizer, or by passing the cells through a 27-gauge needle.

4. Proceed to the differential centrifugation steps ("Preparation of ER from animal tissues" section, Steps 6-7).

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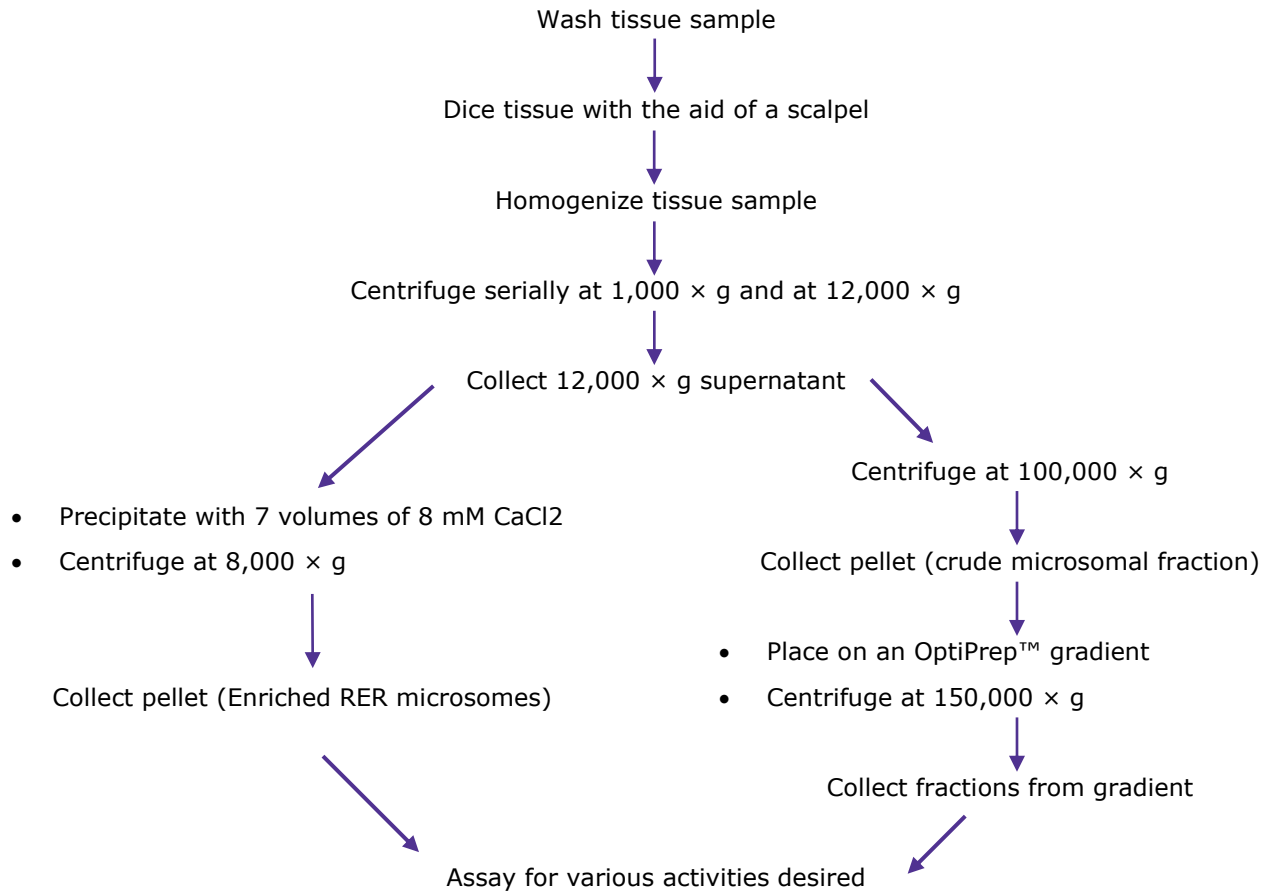
Appendix I. Preparations of Endoplasmic Reticulum (ER)



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