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ProductInformation

Endoplasmic Reticulum Isolation Kit

Catalog Number **ER0100**
Storage Temperature 2–8 °C

TECHNICAL BULLETIN

Product Description

The endoplasmic reticulum (ER) is a network of interconnected tubules, vesicles, and sacs. They play a role in specialized cellular functions including protein synthesis, sequestering of calcium, production of steroids, storage and production of glycogen, and 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 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] and density gradient purified rough ER (RER) and smooth ER (SER).[6]

The ER isolation process can be monitored by measuring the activity of NADPH cytochrome c reductase (Cytochrome c Reductase Assay Kit, Catalog Number CY0100). The enzyme is an ER membrane protein that is commonly used as an ER marker.[4]

The isolated ER can be used for studying the cytochrome P450 system and xenobiotic metabolism, for studying lipid metabolism, and for the recovery of ER membrane and lumenal proteins. The kit was tested with rat liver, kidney, and brain; mouse liver; rabbit liver, kidney, spleen, heart, and brain; and with Jurkat and HeLa cell lines.

Components

Component	Quantity
Isotonic Extraction Buffer 5× Catalog Number I3533 50 mM HEPES, pH 7.8, with 1.25 M sucrose, 5 mM EGTA, and 125 mM potassium chloride	100 ml
Hypotonic Extraction Buffer 10× Catalog Number H8412 100 mM HEPES, pH 7.8, with 10 mM EGTA and 250 mM potassium chloride	10 ml
Calcium Chloride Solution Catalog Number C2052 2.5 M calcium chloride solution	5 ml
OptiPrep Density Gradient Medium Catalog Number O3028 60% (w/v) solution of iodixanol in water	100 ml
Needle, 4 inch, 20 gauge Catalog Number N4161	1 each

Reagents and Equipment Required But Not Provided

- Protease Inhibitor Cocktail for use with mammalian cell and tissue culture extracts (Catalog Number P8340)
- Dulbecco's Phosphate Buffered Saline (PBS) (Catalog Number D8537)
- Ultrapure water
- Sorvall® RC-5C centrifuge with SS-34 head or equivalent
- Ultracentrifuge with a Kontron 65Ti fixed-angle head or equivalent and 8 ml tubes
- Appropriate homogenizer:
  - Potter-Elvehjem PTFE pestle in glass tube homogenizer - 3 ml (Catalog Number P7734), 8 ml (Catalog Number P7859), 45 ml (Catalog Number P7984)
  - Pellet pestle (Catalog Number Z359947) and Motor for pellet pestle (Catalog Number Z359971)
  - Dounce glass tissue grinder set, 7 ml (Catalog Number 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)

Precautions and Disclaimer
The Endoplasmic Reticulum Isolation Kit is for R&D use only, not for drug, household, or other uses. Please consult the Material Safety Data Sheet for information regarding hazards and safe handling practices.

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 (Catalog Number N4161) and the OptiPrep Density Gradient Medium (Catalog Number 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 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 obtain 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*), in order to obtain RER enriched microsomes.

Option 2 - Centrifugation of the PMF supernatant at 100,000 × *g* to obtain a crude microsomal preparation, which can be either used as is or 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 Preparartion
Note: Use ultrapure water for the reagent preparation.

1. 1× Isotonic Extraction Buffer - 10 mM HEPES, pH 7.8, 250 mM sucrose, 25 mM potassium chloride, and 1 mM EGTA
   Aseptically remove an aliquot of the Isotonic Extraction Buffer 5× (Catalog Number I3533) and dilute it 5-fold with water. Keep the 1× Isotonic Extraction Buffer at 4 °C before use. Just before use add Protease Inhibitor Cocktail for mammalian cells (Catalog Number P8340) to the 1× Isotonic Extraction Buffer at a concentration of 1% (v/v).

   Note: Suggested volumes of 1× Isotonic Extraction Buffer
   - For Option 1 - use a minimal tissue weight of 0.5–1 g and prepare 10 ml of buffer for 0.5–1 g of tissue weight.
   - For Option 2 without gradient separation – use a minimal tissue weight of 2 g and prepare 20 ml of buffer for 2 g of tissue weight.
   - For Option 2 with gradient separation - use a minimal tissue weight of 4 g and prepare 60 ml of buffer: 40 ml for crude microsome preparation and 20 ml for the density gradient separation (when using two 8 ml centrifuge tubes).

2. 1× Hypotonic Extraction Buffer - 10 mM HEPES, pH 7.8, 25 mM potassium chloride and 1 mM EGTA (required for preparation of ER from cultured cells)
   Dilute an aliquot of the Hypotonic Extraction Buffer 10× (Catalog Number H8412) 10-fold with water. Keep the 1× Hypotonic Extraction Buffer at 4 °C before use. Just before use add the Protease Inhibitor Cocktail for mammalian cells (Catalog Number P8340) to the 1× Hypotonic Extraction Buffer at a concentration of 1% (v/v).

   Prepare 3 ml of 1× Hypotonic Extraction Buffer for each ml of packed cells.

3. 8 mM Calcium Chloride Solution - Dilute an aliquot of the 2.5 M $CaCl_2$ Solution (Catalog Number C2052) with water, 0.032 ml of the 2.5 M $CaCl_2$ Solution per 10 ml of water. Keep the 8 mM Calcium Chloride Solution at 4 °C before use.

   Prepare 7.5 ml of the 8 mM Calcium Chloride Solution for each ml of PMF.

4. Optiprep density gradient - For separation in 8 ml centrifuge tubes prepare:
- 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.

A. 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 below 1 g use an 8 ml PTFE pestle in glass tube homogenizer. For samples greater than 1 g use a 45 ml PTFE pestle in 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: This step is performed in order to remove blood from certain tissues (liver). Place the tissue on a paper towel in order to absorb excess liquid and blood clots, if present. Cut the tissue into small pieces (1.5–2 cm) and repeat the wash step.
3. Blot the tissue on a paper towel and weigh.
4. Cut the tissue, with the aid of a scalpel and glass plate, into small slices (0.3–0.5 cm). Transfer the slices into a suitable glass homogenizer. Add 3.5 ml of the 1× Isotonic Extraction Buffer per gram of tissue and homogenize the sample by an overhead motor (~200 rpm). Ensure total homogenization of the sample by moving the pestle up and down at least 7 times. 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 and add to the previous homogenate. Keep the homogenate on ice.
6. Centrifuge the homogenate at 1,000 × *g* for 10 minutes at 4 °C. Carefully remove the thin floating lipid layer by aspiration, being careful not to aspirate the post nuclear supernatant. Transfer the supernatant to another centrifuge tube using a pipette and discard the pellet.
7. Centrifuge at 12,000 × *g* for 15 minutes at 4 °C. Carefully remove the thin floating lipid layer by aspiration, being careful not to aspirate the post mitochondrial supernatant. Transfer the supernatant to another tube using a pipette and discard the pellet. **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 calcium chloride)
Perform this 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 times the volume of the PMF (V ml) (instructions in Reagent Preparation section).
3. Transfer the PMF to a beaker (a size of 10 times the volume of the PMF) containing a suitable magnetic spinbar.
4. Add a volume of 8 mM Calcium Chloride Solution equivalent to 7.5 times 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 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 and 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 (less than 0.8 ml) use a pellet pestle in a microcentrifuge tube. If the volume is larger than 0.8 ml, use a 3 ml PTFE pestle in 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 this 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 and discard.
3. The pellet is difficult to suspend. Therefore, transfer it 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) and transfer the liquid to the homogenizer vessel. If the volume of the suspension is small (less than 0.8 ml) use a pellet pestle in a microcentrifuge tube. If the volume is larger than 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 (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 is based on the 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 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 x 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, in order to facilitate the 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 and 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 this procedure at 4°C

1. Dilute the crude microsomal sample (Procedure A, 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. Take an 8 ml ultracentrifuge tube and place 2 ml of the 30% Optiprep solution at the bottom.
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 and 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 and clamp it 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 (insert the end of the needle to the bottom of the first calibration line and withdraw the aliquot). Record the volume withdrawn, as there may be variations between samples. Transfer the fraction to a microcentrifuge tube and close the tube.
10. Continue to withdraw fractions in the same fashion by moving the needle to the bottom of the next calibration line. Transfer each fraction to a new microcentrifuge tube and 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's 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 the Cytochrome c Reductase (NADPH) Assay Kit (Catalog Number CY0100).

    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.

B. 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 (Procedure A, steps 1-7), which may have to be altered as follows:

For adherent cells (such as HeLa cells, >2 x 10^8 cells):

1. Detach cells using conventional tissue culture methods.
2. Centrifuge the cells at 600 × *g* for 5 minutes and remove the supernatant by aspiration.
3. Wash the cells with 10 volumes of PBS and 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 times the PCV and 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 times the "new" PCV and transfer to a 7 ml Dounce homogenizer.
8. Break the cells with 10 strokes of the Dounce homogenizer and then proceed to the differential centrifugation steps (Procedure A, steps 6-7).

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

1. Perform steps 2-4 of the procedure for adherent cells.
2. Suspend the cells in a volume of 1× Isotonic Extraction Buffer equivalent to 4 times the 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 (Procedure A, steps 6-7).

References

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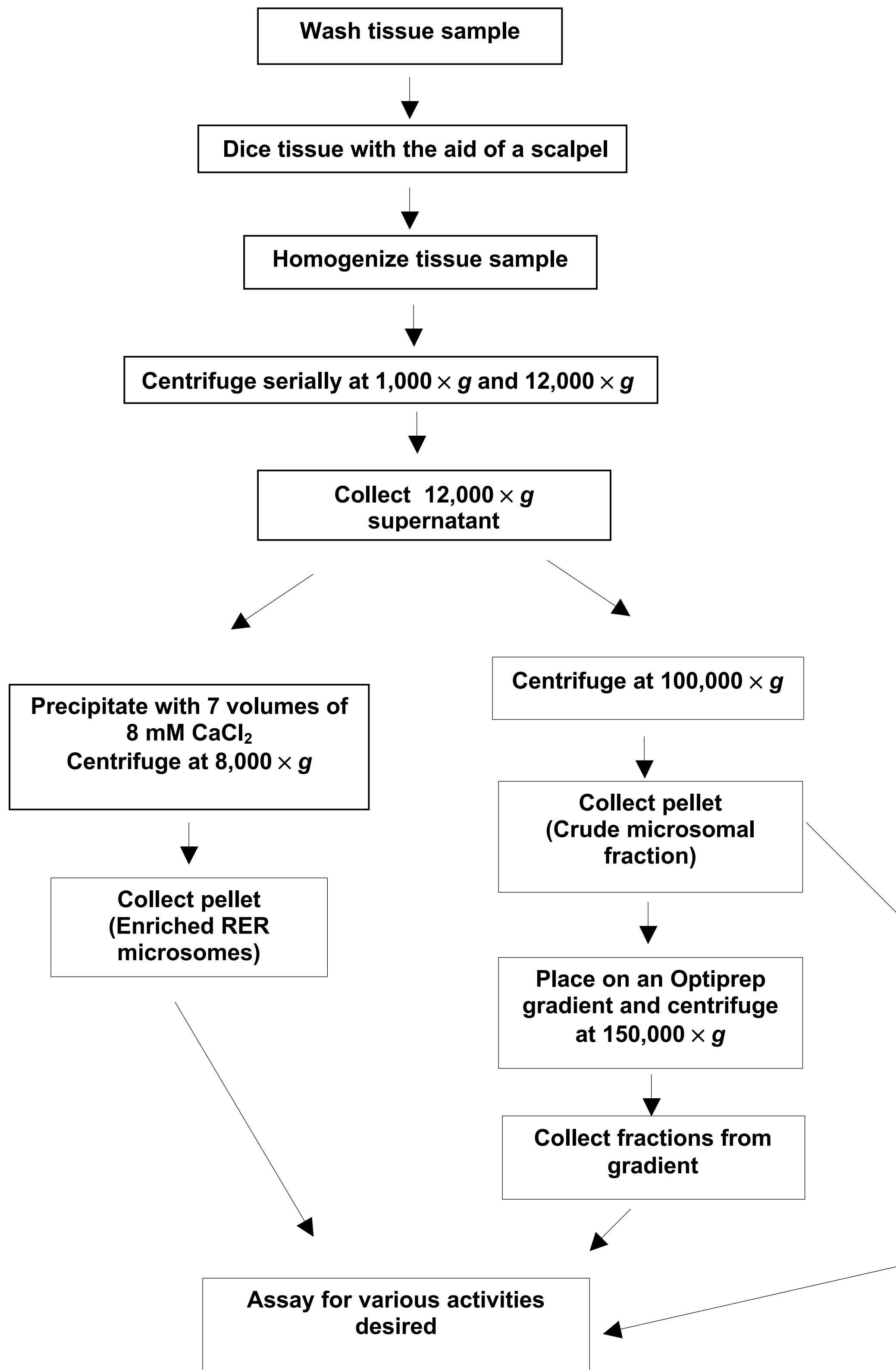
Appendix I



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