Product Information

Peroxisome Isolation Kit

To isolate peroxisomes from tissues and cells

PEROX1

Product Description

Peroxisomes are organelles that are ubiquitously distributed in most prokaryotic and eukaryotic cells. They are spherical particles with a diameter of 0.2-1.0 µm. Peroxisomes are sub-compartmentalized with a unit membrane that surrounds a matrix compartment (similar to mitochondria) which contains the bulk of the peroxisomal enzymes.

Peroxisomes are most abundant in the mammalian liver and kidney.[1-3] In hepatocytes and kidney cortex epithelial cells, peroxisomes are large (0.3-1.5 µm). In other cells such as fibroblasts or smooth muscle cells, peroxisomes are small (0.05-0.2 µm). Isolated peroxisomes may be used to study lipid β-oxidation,[4] amino acid metabolism,[5] and biosynthesis of ether-linked glycerolipids[6] and bile acids.[7,8]

The Peroxisome Isolation Kit provides all necessary reagents and a comprehensive protocol for isolating peroxisomes from animal tissues and from cultured cells by differential centrifugation followed by density gradient centrifugation. The kit was tested with rat liver and kidney, rabbit liver, and with HEK293 and HepG2 cell lines.

The presence of peroxisomes can be determined by measuring the activity of the peroxisomal enzyme marker, catalase (such as with the Catalase Assay Kit, Cat. No. CAT100). In addition, the detection of peroxisomal membrane proteins and their degree of enrichment in the purified fraction can be performed by immunoblotting, using antibodies specific for the peroxisomal membrane-specific marker PMP70 (Cat. No. P0497). Separation of peroxisomes from mitochondria can be determined by measuring the activity of the mitochondrial enzyme marker, cytochrome c oxidase (such as with the Cytochrome c Oxidase Assay Kit, Cat. No. CYTOCOX1).

Several dissertations[10-14] have cited use of the PEROX1 kit in their protocols.

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.

Storage/Stability

The kit is shipped on wet ice. Long-term storage at 2-8 °C is recommended. Upon receiving the kit, the Protease Inhibitor Cocktail (Cat. No. P8340) should be stored at –20 °C and the OptiPrep™ Density Gradient Medium (Component O3028) should be stored at room temperature. When stored properly, the components in this kit are stable for 24 months.

Components

The kit is sufficient for the preparation of peroxisomes from 50 g of tissue or ~20 mL of packed cells.

- Peroxisome Extraction Buffer 5×, 100 mL (Component P7247): 25 mM MOPS (pH 7.65), with 1.25 M sucrose, 5 mM EDTA, and 0.5% ethanol
- OptiPrep™ Dilution Buffer 20×, 10 mL (Component O4889): 100 mM MOPS (pH 8.0), with 20 mM EDTA and 2% ethanol
- Protease Inhibitor Cocktail for use with mammalian cell and tissue extracts, 5 mL (Cat. No. P8340)
- OptiPrep™ Density Gradient Medium, 100 mL (Component O3028): 60% (w/v) solution of iodixanol in water

Reagents and Equipment Required

(Not provided)

- Refrigerated superspeed floor centrifuge with SS-34 head or equivalent

- Ultracentrifuge and a fixed angle head suitable for centrifugation at 100,000 × g with 8 mL sample volume per tube
- Ultrapure water
- Dulbecco's Phosphate Buffered Saline (D-PBS, Cat. No. D8537)
- Microcentrifuge tubes
- Pasteur pipettes

Equipment Required for Peroxisome Preparation from Tissue Extracts

- Homogenizers:
  - For initial homogenization: Tissue homogenizer with S25N 18G head or equivalent
  - For homogenization of 25,000 × g pellet: Overhead electric motor together with Potter-Elvehjem PTFE pestle in glass tube homogenizer:
    - 3 mL (Cat. No. P7734), or
    - 8 mL (Cat. No. P7859)
- Scalpel
- Forceps
- Glass plate
- 40 mL polypropylene centrifuge tubes

Equipment and Reagents Required for Peroxisome Preparation from Cell Culture Extracts

- Trypsin-EDTA (for removal of adherent cells from vessel surface, Cat. No. T4049)
- Trypan Blue (Cat. No. T8154)
- Refrigerated tabletop centrifuge
- Adaptor for microcentrifuge tubes in refrigerated superspeed floor centrifuge
- 14 mL centrifuge tubes with adapter for SS-34 head
- Homogenizers:
  - For initial homogenization:
    - 7 mL Dounce glass tissue grinder (Cat. No. T0566)
    - Small clearance pestle (Cat. No. P1235)
  - For homogenization of 25,000 × g pellet:
    - Pellet pestle
    - Motor for pellet pestle (Cat. No. Z359971)

Additional Equipment and Reagents Required to Measure Enzyme Activities

- Spectrophotometer with a 1 mL cuvette
- Catalase Assay Kit (Cat. No. CAT100)
- Cytochrome c Oxidase Assay Kit (Cat. No. CYTOCOX1)

Preparation Instructions

It is recommended to use ultrapure (17 MΩ•cm or equivalent) water when preparing the reagents.

1× Peroxisome Extraction Buffer

1. Dilute an aliquot of the Peroxisome Extraction Buffer 5x (Component P7247) 5-fold with ultrapure water. Keep the diluted Peroxisome Extraction Buffer at 4 °C until use.
2. Just before use, add Protease Inhibitor Cocktail for mammalian cell and tissue extracts (Cat. No. P8340) to the diluted Peroxisome Extraction Buffer at a concentration of 1% (v/v). The diluted Peroxisome Extraction Buffer with the 1% (v/v) Protease Inhibitor Cocktail is the 1× Peroxisome Extraction Buffer.
3. Suggested volumes of 1× Peroxisome Extraction Buffer are as follows:
   3.1. For tissue extracts: use a minimal tissue weight of 4 g and prepare 25 mL of buffer.
   3.2. For cell culture extracts: prepare a minimum of 2 × 10^8 cells and 10 mL of buffer.

1× OptiPrep™ Dilution Buffer

1. Dilute an aliquot of the OptiPrep™ Dilution Buffer 20× (Component O4889) 20-fold with water. Keep the 1× OptiPrep™ Dilution Buffer at 4 °C until use.
2. Suggested volumes of 1× OptiPrep™ Dilution Buffer are as follows:
   2.1. For tissue extracts from 4 g tissue: 25 mL
   2.2. For cell culture extracts of at least 2 × 10^8 cells: 20 mL

OptiPrep™ Density Gradient Medium Solutions

For separation in 8 mL centrifuge tubes, prepare:

- 27.5% OptiPrep™ Solution:
  - Mix 4.58 mL of OptiPrep™ Density Gradient Medium [60% (w/v)] with 5.42 mL of 1× OptiPrep™ Dilution Buffer.
  - Mix well by inversion.
- 20% OptiPrep™ Solution:
  - Mix 3.33 mL of OptiPrep™ Density Gradient Medium [60% (w/v)] with 6.67 mL of 1× OptiPrep™ Dilution Buffer.
  - Mix well by inversion.

Procedure

Peroxisomes with different degrees of purity can be easily prepared from animal tissues using a simple method of homogenization followed by differential centrifugation. Tissue culture cells need to be homogenized in a Dounce homogenizer and the degree of breakage carefully followed by Trypan Blue staining. Excess breakage will cause widespread damage to the peroxisomes.

The serial centrifugations include:

- Low-speed centrifugation (1000 × g)
- Low-speed centrifugation (2000 × g)
- Medium-speed centrifugation (25,000 × g)

The serial centrifugations remove nuclei, cell debris, mitochondria, and lipids to obtain a crude peroxisomal fraction (CPF). The CPF pellet is the starting material for the preparation of purified peroxisomes.

A highly enriched peroxisome fraction can be prepared from the CPF pellet by efficient removal of the other organelles using an OptiPrep™ density gradient:

- The CPF suspended in 22.5% OptiPrep™ is layered between 27.5% and 20% OptiPrep™ layers.
- The sample is centrifuged at 100,000 × g for 1.5 hours. The purified peroxisome fraction is collected from the bottom of the tube.[9]
- **Note**: If an ultracentrifuge is not available, it is possible to centrifuge the same tubes in a refrigerated superspeed floor centrifuge for 3.5 hours at 45,000 × g (19,400 rpm in an SS-34 head).

A flow diagram for the various peroxisome preparations is shown in the Appendix.

Preparation of Crude Peroxisomal Fraction (CPF)

From Animal Tissue (~4 grams of tissue)

Perform the procedure at 4 °C. All solutions and equipment should be cooled before use. Homogenize the samples using a tissue homogenizer with a S25N 18G head.

1. Use a fresh tissue sample from an animal that was starved overnight and sacrificed the next morning.
2. Wash the tissue sample three times with 10-15 mL of ice-cold PBS:
   2.1. Place the tissue in a dish.
   2.2. Shake gently for a few minutes.
   2.3. Remove the PBS.
   2.4. Place the tissue on a paper towel to absorb excess liquid and blood clots, if present.
   2.5. Cut the tissue into small pieces (1.5-2 cm).
   2.6. Repeat the Wash step.

   Note: This sequence of steps is performed to remove excess blood from certain tissues, such as liver.
3. Blot the tissue on a paper towel. Weigh.
4. Cut the tissue on a glass plate with the aid of a scalpel into small slices (0.3-0.5 cm in width).
   4.1. Transfer the slices into a 40 mL polypropylene centrifuge tube.
   4.2. Add 4 volumes of the 1× Peroxisome Extraction Buffer per gram of tissue (such as 16 mL per 4 grams).
   4.3. Homogenize the sample as follows:
   - Homogenize at 8,000 rpm for 5 seconds.
   - Perform 2 additional homogenizations at 9,500 rpm for 5 seconds each.
5. Wash the homogenizer head with 1 mL of the 1× Peroxisome Extraction Buffer.
   5.1. Add the wash 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. Remove the floating lipid layer by aspiration.
   6.2. Transfer the supernatant to another centrifuge tube using a pipette.
   6.3. Discard the pellet. This step removes nuclei and other cell debris.

   Note: To monitor the degree of purification following the different centrifugation steps, it is recommended to save a sample (100-200 µL) of the 1,000 × g supernatant for subsequent assays.
7. Centrifuge at 2,000 × g for 10 minutes at 4 °C.
   7.1. Remove the floating lipid layer by aspiration.
   7.2. Transfer the supernatant to another centrifuge tube using a pipette.
   7.3. Discard the pellet. This step removes heavy mitochondria.
8. Centrifuge at 25,000 × g for 20 minutes at 4 °C.
   8.1. Aspirate off the supernatant liquid.
   8.2. Resuspend the pellet in a minimal volume of 1× Peroxisome Extraction Buffer. It is recommended to use 0.4 mL per gram of original tissue (such as 1.6 mL per 4 grams).

The suspension obtained in Step 8 is the Crude Peroxisomal Fraction (CPF) and contains a mixture of light mitochondria, lysosomes, peroxisomes, and endoplasmic reticulum (ER).

Note: The CPF may be kept overnight at 4 °C and then separated the next day on a density gradient.

From Cell Cultures (~2 × 10^8 cells)

Perform the procedure at 4 °C. All solutions and equipment should be cooled before use. Homogenize the samples using a 7 mL Dounce glass tissue grinder (Cat. No. T0566) with a small clearance pestle (Cat. No. P1235).

Note: This procedure requires a relatively large amount of cells, 1-2 mL packed cell volume representing at least 2 × 10^8 cells.

1. Wash the cells:

For adherent cells:

   1.1. Wash cells with PBS to remove the serum (~8 mL per 100 cm^2).

   1.2. Detach the cells from the surface with Trypsin or Trypsin-EDTA.

   1.3. Pour the trypsinized cells into growth medium containing 10% Fetal Bovine Serum (Cat. No. F6178) (4 mL per 100 cm^2). This quenches the proteolytic action of the trypsin.

For cells in suspension, start from Step 2 below.

2. Centrifuge the cell suspension for 5 minutes at 250 × g.

   2.1. Discard the supernatant.

   2.2. Resuspend the cells in 40 mL of PBS.

3. Centrifuge the resuspended cells for 5 minutes at 250 × g.

   3.1. Discard the supernatant.

   3.2. Wash the cells twice in this fashion. The packed cell volume (PCV) should be 1.5-3 mL.

4. Add 2.7 PCV of 1× Peroxisome Extraction Buffer. Vortex to achieve an even suspension.

5. Break the cells in a 7 mL Dounce homogenizer (Cat. No. T0566) using Pestle B (small clearance). This may necessitate splitting the fraction into two portions.

6. After every 5 strokes with the pestle, stain an aliquot of cells with Trypan Blue.

   6.1. Examine under a microscope to assess the degree of breakage.

   6.2. Normally 15-25 strokes are sufficient to achieve 80-85% breakage. Do not try to achieve higher breakage levels, as this will lead to severe damage of the peroxisomes.

7. Centrifuge the sample at 1,000 × g for 10 minutes.

8. Transfer the supernatant to a new centrifuge tube.

   Note: To monitor the degree of purification following the different centrifugation steps, it is recommended to save a sample (100-200 µL) of the 1,000 × g supernatant for subsequent assays.

9. Centrifuge the sample at 2,000 × g for 10 minutes.

10. Transfer the supernatant to a new centrifuge tube.

11. Centrifuge the sample at 25,000 × g for 20 minutes in microcentrifuge tubes.

12. Remove the supernatant liquid and collect the pellet in a minimal volume of 1× Peroxisome Extraction Buffer (~0.4 mL per 10^8 cells).

13. Uniformly suspend the pellet in a single centrifuge tube by using a pellet pestle.

The suspension obtained in Step 13 is the Crude Peroxisomal Fraction (CPF) and contains a mixture of light mitochondria, lysosomes, peroxisomes, and endoplasmic reticulum.

Note: The CPF may be kept overnight at 4 °C and then separated the next day on a density gradient.

Isolation of Peroxisomes on a Density Gradient

The objective of density gradient centrifugation is to obtain enriched peroxisomes separated from other organelles, such as mitochondria.

This procedure uses an 8 mL ultracentrifuge tube.

1. Dilute the CPF ("From Animal Tissue", Step 8, or "From Cell Cultures", Step 13) as follows:

   1.1. To 1.2 mL of the CPF, add 1.69 mL of the OptiPrep™ Density Gradient Medium and 1.61 mL of the 1× OptiPrep™ Dilution Buffer.

   1.2. This yields a diluted CPF sample with a final volume of 4.5 mL and an OptiPrep™ concentration of 22.5%.

   1.3. If the CPF sample is not properly diluted, it will tend to drop into the lower density layer.

2. Place 2 mL of the 27.5% OptiPrep™ Solution into an 8 mL ultracentrifuge tube using a Pasteur pipette.

   2.1. Then overlay with 4.0 mL of the diluted CPF sample.

   2.2. Overlay the sample with 2 mL of the 20% OptiPrep™ Solution.

   Note: Overlay the different layers by carefully applying the solutions onto the wall of the tube.

3. Close the tube. Ensure that this tube is balanced against another tube.

4. Centrifuge for 1.5 hours at 100,000 × g.

   4.1. When de-accelerating the centrifuge, brake to 800 rpm.

4.2. Then let it to come to a stop without use of the brake. The tube will show:

- A cloudy, floating layer on the top of the gradient that mainly consists of ER and lysosomes
- A ring at the 22.5%/27.5% interface that is mitochondria, and:
- A slightly floating pellet in the bottom layer.

5. Carefully aspirate off the top layers, including the ring at the 22.5%/27.5% interface. This step is important to obtain a fraction that is as free as possible of mitochondria.
6. Withdraw the bottom layer containing the **purified peroxisomes**.

Note: The purified peroxisomes may also be kept at 4 °C for up to 24 hours with little degradation. If an immunoblot of specific proteins in the peroxisome is desired, it is advisable to add the sample buffer immediately after preparation of the purified fraction, and then to freeze the sample.

Analysis of Peroxisome Purification

The degree of purification of the peroxisomes can be evaluated by comparing the protein concentration, catalase (peroxisomal marker) and cytochrome c oxidase (mitochondrial marker) activities of the purified peroxisomes to that of the 1000 × g supernatant. The ratio of catalase activity to cytochrome c oxidase activity in the purified peroxisomes should be at least 15-30 times greater than that found in the 1,000 × g supernatant.

The purified peroxisomes can be assayed according to the following guidelines. Catalase activity **must** be determined by a **colorimetric** method, since OptiPrep™ interferes with the UV method.

Protein concentration by Bradford method

1. Dilute 10-fold.
2. Use 10-40 µL per test.

Catalase activity

1. For tissues:
   1.1. Dilute 20 to 100-fold.
   1.2. Use 2.5-5.0 µL per test.
2. For cell extracts:
   2.1. Dilute 4-fold.
   2.2. Use 2.5-5.0 µL per test.

Cytochrome c oxidase activity

1. Dilute 3-fold.
2. Use 20-100 µL per test.

The 1000 × g supernatant can be assayed according to the following guidelines:

For protein determination

1. Dilute 100-fold.
2. Use 20-40 µL per test.

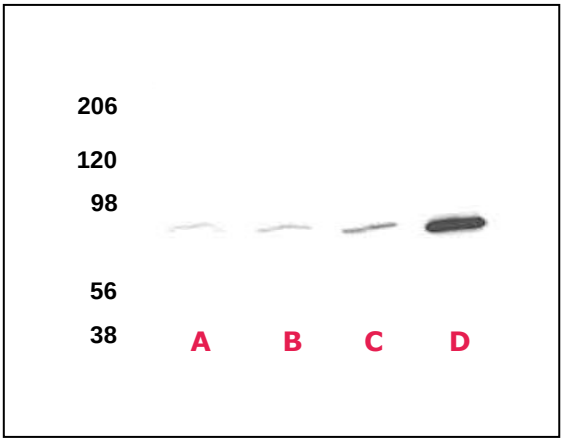
Catalase activity

1. For tissues:
   1.1. Dilute 100-fold.
   1.2. Use 2.5-5.0 µL per test.
2. For cell extracts:
   2.1. Dilute 6-fold.
   2.2. Use 2.5-5.0 µL per test.
3. Cytochrome c oxidase activity:
   3.1. Dilute 3-fold.
   3.2. Use 5-10 µL per test.

Results

An immunoblot of the purified fractions using the anti-PMP70 antibody (Cat. No. P0497), an antibody against peroxisomal membrane protein 70 (PMP70), is shown below in Figure 1.

Figure 1. Enrichment of peroxisomes in isolated fractions detected by anti-PMP70 antibody



Rat liver peroxisomes were purified according to the isolation procedure. Samples of 0.2 µg protein from the different purification stages/fractions were run on 7.5% SDS-PAGE. Immunoblotting was performed using anti-PMP70 at a dilution of 1:2000.

- Lane A: 1,000 × g supernatant
- Lane B: The endoplasmic reticulum fraction from the OptiPrep™ gradient
- Lane C: The fraction containing mainly mitochondria (with some peroxisomes) from the OptiPrep™ gradient
- Lane D: The purified peroxisome fraction from the OptiPrep™ gradient.

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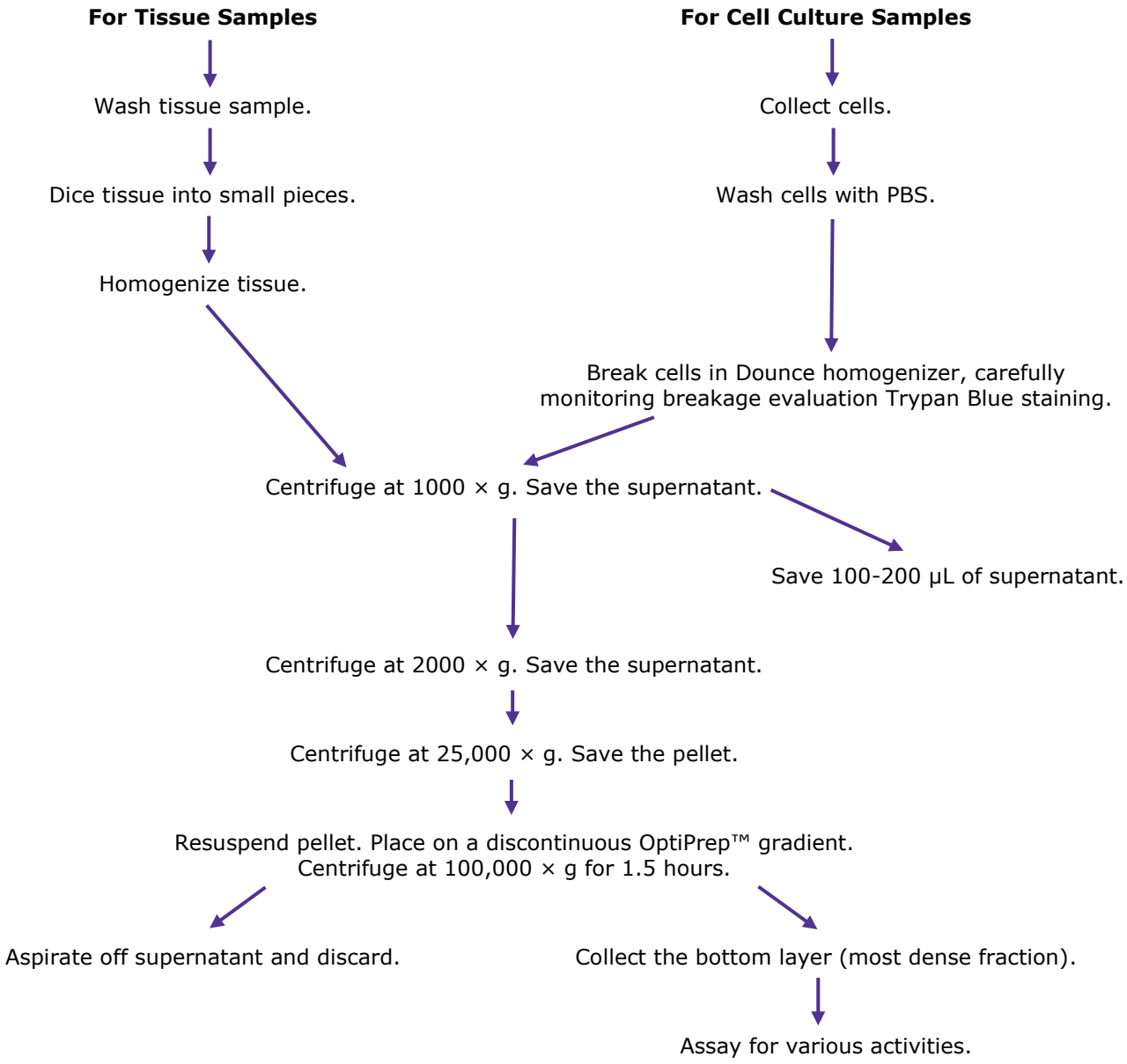
Appendix. Flowchart of Peroxisome Preparation



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