Peroxisome Isolation Kit

Cat. No. PEI-328K **Lot. No.** (See product label)

SPECIFICATION

Product Overview	This kit has been used for preparation of peroxisomes from rat liver, rat kidney and rabbit liver as well as HEK293 and HepG2 cells.
Usage	The kit is sufficient for the preparation of peroxisomes from 50 g of tissue or ~20 ml of packed cells
Storage	The kit is shipped on wet ice and storage at 2-8 centigrade is recommended. Upon receiving the kit, the Protease Inhibitor Cocktail should be stored at -20 centigrade and the OptiPrep Density Gradient Medium should be stored at room temperature. When stored properly, the components in this kit are stable for 24 months.
Kit Components	Peroxisome Extraction Buffer 5X 100 ml Optiprep Dilution Buffer 20X 10 ml Protease Inhibitor Cocktail for use with 5 ml OptiPrep Density Gradient Medium 100 ml
Materials Required but Not Supplied	• Sorvall RC-5C centrifuge with SS-34 head or equivalent • Ultracentrifuge and a fixed angle head suitable for centrifugation at 100,000 x g with 8 ml sample volume per tube • Ultrapure water • Dulbecco's Phosphate Buffered Saline (PBS) • Microcentrifuge tubes • Pasteur pipettes

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Preparation	It is recommended to use ultrapure (17 MΩcm or equivalent) water when preparing the reagents. 1x Peroxisome Extraction Buffer - Dilute an aliquot of the Peroxisome Extraction Buffer 5x 5-fold with ultrapure water. Keep the diluted Peroxisome Extraction Buffer at 4 centigrade until use. Just before use add Protease Inhibitor Cocktail for mammalian cell and tissue extracts (Product Code P 8340) 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 1x Peroxisome Extraction Buffer. Suggested volumes of 1x Peroxisome Extraction Buffer: • For tissue extracts: use a minimal tissue weight of 4 g and prepare 25 ml of buffer. • For cell culture extracts: prepare a minimum of 2 x 108 cells and 10 ml of buffer. 1x Optiprep Dilution Buffer - Dilute an aliquot of the Optiprep Dilution Buffer 20x 20-fold with water. Keep the 1X Optiprep Dilution Buffer at 4 centigrade until use. Suggested volumes of 1X Optiprep Dilution Buffer: • For tissue extracts from 4 g tissue: 25 ml. • For cell culture extracts of at least 2 x 108 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 1x 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 1X Optiprep Dilution Buffer. Mix well by inversion.
Separation Protocol	Peroxisomes with different degrees of purity can be easily prepared from animal tissues using a simple method of homogenization with the aid of an UltraTurrax T25 tissue homogenizer 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 x g)
• Low speed centrifugation (2000 x g)
• Medium speed centrifugation (25,000 x 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, and centrifuged at 100,00 x g for 1.5 hours The purified peroxisome fraction is collected from the bottom of the tube.
Note: If an ultracentrifuge is not available it is possible to centrifuge the same tubes in a Sorvall RC-5C centrifuge for 3.5 hours at 45,000 x g (19,400 rpm in an SS-34 head).
A flow diagram for the various peroxisome preparations is shown in the Appendix.

A. Preparation of Crude Peroxisomal Fraction (CPF) from Animal Tissue (~4 grams of tissue)
Perform the procedure at 4 centigrade. All solutions and equipment should be cooled before use. Homogenize the samples using an UltraTurrax T-25 tissue homogenizer with 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 by placing the tissue in a dish, shaking gently for few minutes, and removing the PBS. 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.
Note: This step is performed in order to remove excess blood from certain tissues

(e.g. liver).
3. Blot the tissue on a paper towel and 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). Transfer the slices into a 40 ml polypropylene centrifuge tube. Add 4 volumes of the 1x Peroxisome Extraction Buffer per gram of tissue (i.e.16 ml per 4 grams), and homogenize the sample as follows: Homogenization at 8,000 rpm for 5 seconds followed by 2 additional homogenizations at 9,500 rpm for 5 seconds each.
5. Wash the homogenizer head with 1 ml of the 1x Peroxisome Extraction Buffer and add the wash to the previous homogenate. Keep the homogenate on ice.
6. Centrifuge the homogenate at 1,000 x g for 10 minutes at 4 centigrade. Remove the floating lipid layer by aspiration, transfer the supernatant to another centrifuge tube using a pipette, and 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 x g supernatant for subsequent assays.
7. Centrifuge at 2,000 x g for 10 minutes at 4 centigrade. Remove the floating lipid layer by aspiration, transfer the supernatant to another centrifuge tube using a pipette, and discard the pellet. This step removes heavy mitochondria.
8. Centrifuge at 25,000 x g for 20 minutes at 4 centigrade. Aspirate off the supernatant liquid and resuspend the pellet in a minimal volume of 1x Peroxisome Extraction Buffer. It is recommended to use 0.4 ml per gram of original tissue (i.e. 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 centigrade and then separated the next day on a density gradient.

B. Preparation of Crude Peroxisomal Fraction (CPF) from cell cultures (~2 x 10^8

cells)

Perform the procedure at 4 centigrade. All solutions and equipment should be cooled before use. Homogenize the samples using a 7 ml Dounce glass tissue grinder with small clearance pestle.

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

1. Wash the cells For adherent cells:

• Wash cells with PBS to remove the serum (~8 ml per 100 cm2).

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

• Pour the trypsinized cells into growth medium containing 10% Fetal Bovine Serum (4 ml per 100 cm2). This quenches the proteolytic action of the trypsin. For cells in suspension, start with step 2.

2. Centrifuge the cell suspension for 5 minutes at 250 x g (~1,200 rpm in a Sorvall RT-6000B centrifuge). Discard the supernatant and resuspend the cells in 40 ml of PBS.

3. Centrifuge the resuspended cells for 5 minutes at 250 x g and discard the supernatant. Wash the cells twice in this fashion. The packed cell volume (PCV) should be 1.5-3 ml.

4. Add 2.7 PCV of 1X Peroxisome Extraction Buffer and vortex to achieve an even suspension.

5. Break the cells in a 7 ml Dounce homogenizer 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 and examine under a microscope to assess the degree of breakage. 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 x 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 x g supernatant for subsequent assays.
9. Centrifuge the sample at 2,000 x g for 10 minutes.
10. Transfer the supernatant to a new centrifuge tube.
11. Centrifuge the sample at 25,000 x g for 20 minutes in Eppendorf tubes using a special adaptor for the Sorvall centrifuge.
12. Remove the supernatant liquid and collect the pellet in a minimal volume of 1X Peroxisome Extraction Buffer (`~0.4 ml per 10^8 cells`).
13. Uniformly suspend the pellet in a single Eppendorf 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 centigrade and then separated the next day on a density gradient.
C. Isolation of Peroxisomes on a Density Gradient The objective of density gradient centrifugation is to obtain enriched peroxisomes separated from other organelles, e.g. mitochondria. This procedure uses an 8 ml ultracentrifuge tube.
1. Dilute the CPF (section A, step 8 or section B, step 13) as follows: to 1.2 ml of the CPF (crude peroxisomal fraction) add 1.69 ml of the OptiPrep Density Gradient Medium and 1.61 ml of the 1X Optiprep Dilution Buffer. This yields a diluted CPF sample with a final volume of 4.5 ml and an OptiPrep concentration of 22.5%. 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 and then overlay with 4.0 ml of the diluted CPF sample. 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 and ensure that it is balanced against another tube.

4. Centrifuge for 1.5 hours at 100,000 x g. When deaccelerating the centrifuge, brake to 800 rpm and 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 in order 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 centigrade 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.
C. 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 x 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 x g supernatant.
D. Catalase activity must be determined by a colorimetric method since Optiprep interferes with the UV method.
The purified peroxisomes can be assayed according to the following guidelines:
a. Protein concentration by Bradford method: Dilute 10-fold and use 10-40 µper test.
b. Catalase activity: For tissues, dilute 20 to 100-fold and use 2.5-5.0 µper test. For cell extracts, dilute 4-fold and use 2.5-5.0 µper test.
c. Cytochrome c oxidase activity: Dilute 3-fold and use 20-100 µper test.

The 1000 x g supernatant can be assayed according to the following guidelines:
a. For protein determination: Dilute 100-fold and use 20-40 µper test.
b. Catalase activity: For tissues, dilute 100-fold and use 2.5-5.0 µper test. For cell

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	extracts, dilute 6-fold and use 2.5-5.0 µper test. c. Cytochrome c oxidase activity: Dilute 3-fold and use 5-10 µper test.

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