

Lysosome Isolation Kit

Cat. No. LYI-323K **Lot. No.** (See product label)

SPECIFICATION

Product Overview	The Lysosome Isolation Kit provides a method for isolating lysosomes from animal tissues and from cultured cells by differential centrifugation followed by density gradient centrifugation and/or calcium precipitation.
Usage	1 Kit is sufficient for 20 mL (packed cells) or sufficient for 25 g (tissue).
Storage	After 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. All the other components in this kit should be stored at 2–8 centigrade. The components are stable for 24 months when stored unopened
Kit Components	Extraction Buffer 5×100 ml Optiprep Dilution Buffer 20×20 ml Protease Inhibitor Cocktail for use with mammalian cell and tissue extracts 5 ml OptiPrep Density Gradient Medium 60% (w/v) solution of iodixanol in water 100 ml Neutral Red Reagent 1 ml Calcium Chloride Solution 2.5 M calcium chloride solution 1 ml Sucrose Solution, 2.3 M 25 ml
Materials Required but Not Supplied	· Sorvall RC-5C centrifuge with SS-34 head or equivalent · Ultracentrifuge with SW50.1 head or equivalent and 5 ml tubes · Microcentrifuge · Microcentrifuge tubes · Ultrapure water

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- Dulbecco's Phosphate Buffered Saline (PBS)
- Pasteur pipettes
- FinntipFlex 1,000 pipette tip
- Bradford Reagent for protein measurements

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

250 mM Calcium Chloride Solution - Dilute an aliquot of the 2.5 M calcium chloride solution 10-fold with ultrapure water.

1×Extraction Buffer - Dilute an aliquot of the Extraction Buffer 5× 5-fold with ultrapure water. Keep the diluted Extraction Buffer at 2–8 centigrade until use. Just before use add the Protease Inhibitor Cocktail for mammalian cell and tissue extracts to the diluted Extraction Buffer at a final concentration of 1% (v/v). The diluted Extraction Buffer with the 1% (v/v) Protease Inhibitor Cocktail is the 1× Extraction Buffer.

Suggested volumes of 1× Extraction Buffer:

- For tissue extracts: use a minimal tissue weight of 4 g and prepare 25 ml of buffer.
- For cell culture extracts: use a minimum of $2-3 \times 10^8$ cells and prepare 10 ml of

Preparation

buffer. 1× Optiprep Dilution Buffer - Dilute an aliquot of the Optiprep Dilution Buffer 20× 20-fold with ultrapure water. Keep the 1× Optiprep Dilution Buffer at 2-8 centigrade until use.

Suggested volumes of 1× Optiprep Dilution Buffer:

- For tissue extracts from 4 g of tissue: 40 ml
- For cell culture extracts from at least 2×10^8 cells: 30 ml

Optiprep Density Gradient Medium Solutions - Prepare 10 ml of each Optiprep Density Gradient Medium Solution. The OptiPrep Density Gradient Medium supplied with the kit is a 60% (w/v) solution in water. Table 1 shows the final concentration of each Optiprep Density Gradient Medium Solution after dilution. The diluted solutions need to be osmotically balanced with sucrose to ~290 mOsm. These Optiprep Density Gradient Medium Solutions may be kept at 2–8 centigrade for up to 4 weeks, if prepared aseptically.

Separation Protocol

A crude lysosomal fraction can be prepared using a simple method of homogenization followed by differential centrifugation.

The serial centrifugations include:

- Low speed centrifugation ($1,000 \times g$)
 - Medium speed centrifugation ($20,000 \times g$)
- The crude lysosomal fraction (CLF) is obtained after removal of nuclei, cell debris, and fat by the serial centrifugations. The CLF pellet is the starting material for the further preparation of purified lysosomes. Lysosomes may be further purified on a multi-layered step gradient of osmotically balanced Optiprep[®] and/or purified by precipitation of rough ER and mitochondria with calcium ions. A flow diagram for the various preparations of lysosomes is shown in Appendix I.

I. Preparation of Crude Lysosomal Fraction (CLF)

A. From Animal Tissue (~4 gram of tissue) Perform the whole procedure at 2–8 centigrade. All the solutions and equipment should be pre-cooled before use.

Homogenize the samples using an Ultra-Turrax T-25 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 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.
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). Transfer the slices into a 40 ml PP centrifuge tube. Add 4 volumes of the 1× 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 homogenization at 9,500 rpm for two additional 5 second periods.
5. Wash the homogenizer head with 1 ml of the 1× Extraction Buffer and add to the previous homogenate. Keep the homogenate on ice.

6. Centrifuge the homogenate at $1,000 \times g$ for 10 minutes at 2–8 centigrade. Remove the floating fat layer by careful aspiration and transfer the supernatant to another centrifuge tube using a pipette.

7. Rehomogenize the pellet in 2 volumes of 1× Extraction Buffer for 5 seconds at 9,500 rpm. Repeat step 6 and pool the supernatant from this step with the supernatant from step 6. This is defined as the $1,000 \times g$ supernatant. Note: To monitor the degree of purification following the different centrifugation steps, it is recommended to save a sample (~200 ml) of the $1,000 \times g$ supernatant for subsequent assays.

8. Discard the pellet, which contains nuclei and other cell debris.

9. Centrifuge at $20,000 \times g$ for 20 minutes at 2–8 centigrade. Remove the floating fat layer and the supernatant by aspiration

10. Resuspend the pellet in a minimal volume of 1× Extraction Buffer. It is recommended to use 0.8 ml per gram of original tissue (i.e., 3.2 ml per 4 gram). This material is the Crude Lysosomal Fraction (CLF) and contains a mixture of light mitochondria, lysosomes, peroxisomes, and endoplasmic reticulum.

Note: For further purification it is recommended to continue directly to section II, Further Purification of CLF. If necessary, the CLF may be stored overnight at 2–8 centigrade (do not freeze) before continuing.

B. From cultured cells ($\sim 3 \times 10^8$ cells)

Perform the whole procedure at 2–8 centigrade. All the solutions and equipment should be pre-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: 1.5–3 ml packed cell volume, representing at least $2\text{--}3 \times 10^8$ cells.

1. Grow the cells to ~90% confluency.

2. For adherent cells: trypsinize the cells, add growth medium with 10% fetal calf serum, and centrifuge the cells for 5 minutes at $600 \times g$. For cells in suspension perform the centrifugation only. Discard the supernatant.

3. Wash the cells: Resuspend the cells in ice cold PBS, count them, and centrifuge

- for 5 minutes at $600 \times g$ at 2–8 centigrade. Discard the supernatant.
4. Repeat the wash step once again (without the cell count). Discard the supernatant. The packed cell volume (PCV) should be 1.5–3 ml.
5. Add 2.7 PCV of $1\times$ Extraction buffer and vortex to achieve an even suspension.
6. Break the cells in a 7 ml Dounce homogenizer using Pestle B (small clearance). This may necessitate splitting the fraction into two portions.
7. After every 5 strokes with the pestle check the cells under a microscope using Trypan Blue solution staining to ascertain the degree of breakage. Normally 15–25 strokes will suffice to achieve 80–85% of breakage. Do not try to achieve higher levels as this will lead to severe breakage of the lysosomes.
8. Centrifuge the sample at $1,000 \times g$ for 10 minutes.
9. Transfer the supernatant to a new centrifuge tube. Keep a sample (100–200 ml) of the supernatant for subsequent assays.
10. Centrifuge the sample at $20,000 \times g$ for 20 minutes in microcentrifuge tubes.
11. Remove the supernatant liquid and collect the pellet in a minimal volume of $1\times$ Extraction Buffer (~ 0.4 ml per 10^8 cells).
12. Suspend the pellet well in a single microcentrifuge tube by using a pellet pestle (see Equipment). This material is a Crude Lysosomal Fraction (CLF) containing a mixture of mitochondria, lysosomes, peroxisomes, and endoplasmic reticulum. Note: For further purification it is recommended to continue directly to section II, Further Purification of CLF. If necessary, the CLF may be stored overnight at 2–8 centigrade (do not freeze) before continuing.

II. Further Purification of CLF

To further enrich the lysosomes in the CLF, the organelles should be separated from each other. Three options are available for further purification. Common to all the options is dilution of the CLF to a solution containing 19% Optiprep Density Gradient Medium Solution with a protein concentration of 5–10 mg of protein/ml for tissues or 0.5–1.0 mg-protein/ml for cell culture extracts. This solution is defined as the Diluted OptiPrep Fraction (DOF). The purification options are as follows:

Option A: Separation of the DOF by density gradient centrifugation ($150,000 \times g$ for 4

hours) on a multi-step OptiPrep gradient. This method allows separation into heavy and light lysosomes with a relatively high yield (>50%).

Option B: Further purification of the fractions obtained from Option A by addition of calcium chloride to a final concentration of 8 mM and low speed ($5,000 \times g$ for 15 minutes) centrifugation. This method will precipitate the rough endoplasmic reticulum and any mitochondria that are in the fraction. There will be a partial loss of lysosome yield with this step.

Option C: Addition of calcium chloride to the DOF to a final concentration of 8 mM and low speed ($5,000 \times g$ for 15 minutes) centrifugation. This method is very quick and allows purification of the sample without the use of an ultracentrifuge; however, the yield is low (<25%).

Option A: Isolation of lysosomes on a density gradient The procedure is for a 5 ml ultracentrifuge tube.

1. Dilute the CLF (see Table 2). Add the components in order from left to right, mixing at each stage. If the sample is not diluted, it will tend to drop into a lower density layer. This solution is defined as the Diluted OptiPrep Fraction (DOF).
2. Build a step gradient (see Table 3) with 27% Optiprep Density Gradient Medium Solution at the bottom and 8% Optiprep Density Gradient Medium Solution at the top of the tube. Use the Optiprep Density Gradient Medium Solutions described in Table 1 (Preparation Instructions).
3. Balance two tubes against each other.
4. Centrifuge 4 hours at $150,000 \times g$ in an SW50.1 rotor. Brake to 800 rpm and then let stop without brake.

Note: If an ultracentrifuge is not available, it is possible to centrifuge the density gradient in 8 ml tubes in a Sorvall RC-5C centrifuge for 12 hours at $45,000 \times g$ (19,400 rpm in an SS-34 head). A similar separation will be observed.

5. The tube will show multiple bands floating in the gradient. Note: Samples from different sources will give different patterns of separation on the OptiPrep density gradient.

6. Withdraw fractions of 0.5–0.7 ml using extra long tips (Finntip Flex 1,000) starting from the top of the gradient, and number them.

7. Assay the purified fractions for:

a. Protein concentration, using the Bradford method: 20 ml per test with 20-fold dilution

b. Acid phosphatase activity: 40–80 ml per test without dilution

c. b-N-Acetylglucosaminidase activity: For cells 10 ml per test without dilution; for tissues 2.5–5 ml per test with 2–4 fold dilution

d. Cytochrome c oxidase activity: 2.5–25 ml per test with 5-fold dilution

e. Cytochrome c reductase activity: 25 ml per test without dilution

f. Catalase activity: for cell extracts 10 ml per test with 2-fold dilution; for tissues 5 ml per test with 10–100 fold dilution.

Note: To monitor the degree of purification following the separation on an OptiPrep density gradient, the catalase activity must be determined by the colorimetric method, since the OptiPrep Density Gradient Medium interferes with the UV method.

8. The 1,000 × g supernatant (step I, A, 7 or I, B, 9) can be assayed in the following manner: a. Protein concentration, using the Bradford method: for tissues 10–40 ml per test with 100-fold dilution; for cell extracts 10–40 ml per test with 10-fold dilution

b. Acid phosphatase activity: for tissues 5–10 ml per test; for cell extracts 10–40 ml per test, both without dilution

c. b-N-Acetylglucosaminidase activity: For tissues 10–20 ml per test; for cells 10 ml per test, both without dilution

d. Cytochrome c oxidase activity: for tissues 5–10 ml per test with 20-fold dilution; for cell extracts 50 ml per test with 2-fold dilution

e. Cytochrome c reductase activity: for tissues 2.5–5 ml per test; for cell extracts 50 ml per test, both without dilution f. Catalase activity: for cell extracts 10 ml per test with 2–3 fold dilution; for tissues 2.5–5 ml per test with 100-fold dilution.

Note: The 1,000 × g supernatant, which does not contain the OptiPrep Density Gradient Medium, may be assayed using either the colorimetric or UV method.

However, for direct comparison of catalase activity in the 1,000 × g supernatant to

purified fractions separated on the OptiPrep density gradient, the colorimetric method must be used in all assays.

Optiprep (%)	Optiprep (ml)	Optiprep Dilution Buffer (ml)	2.3 M Sucrose (ml)	mOsm*
27	4.5	4.9	0.6	312
22.5	3.75	5.63	0.62	304
19	3.17	6.19	0.64	298
16	2.67	6.68	0.65	280
12	2	7.29	0.71	300
8	1.33	7.9	0.77	282

Sample	Optiprep (D1556) (ml)	Optiprep Dilution Buffer (ml)	2.3 M Sucrose (ml)	CLF (ml)	mOsm*
Liver	0.505	0.65	0.03	0.40	280
Jurkat	0.505	0.275	0.00	0.80	300

Optiprep Solution (%)	Volume (ml)	Sample
27	0.8	
22.5	1.0	
19	1.0	Prepared DOF (step 1)
16	1.0	
12	0.9	
8	0.3	