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Product Information

Lysosome Isolation Kit

Catalog Number **LYSISO1**
Storage Temperature 2–8 °C

TECHNICAL BULLETIN

Product Description

Lysosomes are organelles ubiquitously distributed in most eukaryotic cells. They are spherical particles with a diameter of 0.5–1.5 microns. The pH in them is low (~pH 5.0) and they contain many acid hydrolases that take part in protein degradation in the cell. Lysosomes also contain lipases, polysaccharidases, and nucleases. Deficiencies in some of these enzymes lead to specific lysosomal storage diseases,[1,2] such as Tay Sachs, Gaucher, and Hunter diseases. Lysosomes also contribute to maintaining cellular homeostasis and thus, malfunctions in this organelle will have a direct impact on cell behavior and fate.[3] Lysosomes may also be involved in other cellular processes such as Albinism[4] and aging.[5,6]

The Lysosome Isolation Kit provides a procedure for isolating an enriched lysosomal fraction from animal tissues and cultured cells by differential centrifugation followed by density gradient centrifugation and/or calcium precipitation.

The presence of lysosomes can be determined by measuring the acid phosphatase activity with the Acid Phosphatase Assay Kit (Catalog Number CS0740) or the β-N-Acetylglucosaminidase activity with the β-N-Acetylglucosaminidase Assay Kit (Catalog Number CS0780). These two enzymes are considered lysosomal markers, which will show slightly different patterns on an OptiPrep® density gradient.[7] Separation from other organelles can be measured using the appropriate marker detection kits (see Additional equipment and reagents required for measuring enzyme activities).

Components

The kit is sufficient for the preparation of lysosomes from 25 g of tissue or 20 ml of packed cells.

Extraction Buffer 5× Catalog Number E1156	100 ml
Optiprep Dilution Buffer 20× Catalog Number O4889	20 ml
Protease Inhibitor Cocktail for use with mammalian cell and tissue extracts Catalog Number P8340	5 ml
OptiPrep Density Gradient Medium 60% (w/v) solution of iodixanol in water Catalog Number D1556	100 ml
Neutral Red Reagent Catalog Number N2537	1 ml
Calcium Chloride Solution 2.5 M calcium chloride solution Catalog Number C2052	1 ml
Sucrose Solution, 2.3 M Catalog Number S4189	25 ml

Equipment and Reagents Required But Not Provided

- 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
- Dulbecco's Phosphate Buffered Saline (PBS, Catalog Number D8537)
- Pasteur pipettes
- Finntip® Flex 1,000 pipette tip (Catalog Number Z677914)
- Bradford Reagent for protein measurements (Catalog Number B6916)

Equipment required for preparation of lysosomes from tissue extracts

- Homogenizers:
  - For initial homogenization – Tissue homogenizer (Ultra-Turrax® T-25 homogenizer with S25N 18G head or equivalent)
  - Overhead electric motor together with Potter-Elvehjem PTFE pestle in glass tube homogenizer - 3 ml (Catalog Number P7734) or 8 ml (Catalog Number P7859)
- Scalpel, forceps, and glass plate
- 40 ml Polypropylene (PP) centrifuge tubes

Equipment and reagents required for preparation of lysosomes from cell culture extracts

- Trypsin-EDTA (for removal of adherent cells from vessel surface)
- Trypan Blue solution (Catalog Number T8154)
- Sorvall RT-6000B centrifuge
- Adapter for microcentrifuge tubes in Sorvall RC-5C
- 14 ml centrifuge tubes with adapter for SS-34 head
- Homogenizers:
  - 7 ml Dounce glass tissue grinder (Catalog Number T0566) with small clearance pestle (Catalog Number P1235) for cell culture samples
  - Pellet pestle (Catalog Number Z359947) and Motor for pellet pestle (Catalog Number Z359971)

Additional equipment and reagents required for measuring enzyme activities

- Acid Phosphatase Assay Kit (Catalog Number CS0740) - Acid phosphatase is a lysosomal marker.
- β-N-Acetylglucosaminidase Assay Kit (Catalog Number CS0780)
- Cytochrome c Oxidase Assay Kit (Catalog Number CYTOCOX1) - Cytochrome c oxidase is a mitochondrial marker.
- Cytochrome c Reductase Assay Kit (Catalog Number CY0100) - Cytochrome c reductase is an endoplasmic reticulum (ER) marker.
- Catalase Assay Kit (Catalog Number CAT100) - Catalase is a peroxisomal marker.
  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.
- Spectrophotometer with a 1 ml cuvette for measurement of enzyme activities

Precautions and Disclaimer

This product 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.

Preparation Instructions

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

250 mM Calcium Chloride Solution - Dilute an aliquot of the 2.5 M calcium chloride solution (Catalog Number C2052) 10-fold with ultrapure water.

1× Extraction Buffer - Dilute an aliquot of the Extraction Buffer 5× (Catalog Number E1156) 5-fold with ultrapure water. Keep the diluted Extraction Buffer at 2–8 °C until use. Just before use add the Protease Inhibitor Cocktail for mammalian cell and tissue extracts (Catalog Number P8340) 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 buffer.

1× Optiprep Dilution Buffer - Dilute an aliquot of the Optiprep Dilution Buffer 20× (Catalog Number O4889) 20-fold with ultrapure water. Keep the 1× Optiprep Dilution Buffer at 2–8 °C 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 (see Table 1). The OptiPrep Density Gradient Medium (Catalog Number D1556) 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 °C for up to 4 weeks, if prepared aseptically.

Table 1.
Preparation of Optiprep Density Gradient Medium Solutions

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

* These values are representative only and it is recommended to determine the actual values experimentally (should be ~290 mOsm).

Storage/Stability
After receiving the kit, the Protease Inhibitor Cocktail (Catalog Number P8340) should be stored at –20 °C and the OptiPrep Density Gradient Medium (Catalog Number D1556) should be stored at room temperature. All the other components in this kit should be stored at 2–8 °C. The components are stable for 24 months when stored unopened.

Procedure
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 × *g*)
- Medium speed centrifugation (20,000 × *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[8] 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 °C. 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 × *g* for 10 minutes at 2–8 °C. 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 × *g* supernatant.
   Note: To monitor the degree of purification following the different centrifugation steps, it is recommended to save a sample (~200 µl) of the 1,000 × *g* supernatant for subsequent assays.
8. Discard the pellet, which contains nuclei and other cell debris.
9. Centrifuge at 20,000 × *g* for 20 minutes at 2–8 °C. 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 °C (do not freeze) before continuing.

B. From cultured cells (~3×10^8 cells)
Perform the whole procedure at 2–8 °C. All the solutions and equipment should be pre-cooled before use. Homogenize the samples using a 7 ml Dounce glass tissue grinder (Catalog Number T0566) with small clearance pestle (Catalog Number P1235).

Note: This procedure requires a relatively large amount of cells: 1.5–3 ml packed cell volume, representing at least 2–3×10^8 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 × *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 × *g* at 2–8 °C. 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× 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 × *g* for 10 minutes.
9. Transfer the supernatant to a new centrifuge tube. Keep a sample (100–200 µl) of the supernatant for subsequent assays.
10. Centrifuge the sample at 20,000 × *g* for 20 minutes in microcentrifuge tubes.
11. Remove the supernatant liquid and collect the pellet in a minimal volume of 1× 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 °C (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 × *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 × *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 × *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).

Table 2.
Preparation of 19% Diluted Optiprep Fraction (DOF) for Loading on the Gradient.

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

* These values are representative only and it is recommended to determine the actual values experimentally (should be ~290 mOsm).

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).

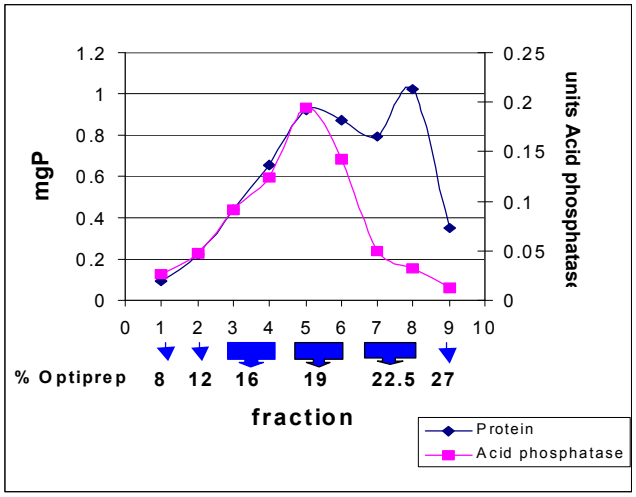
Table 3.
Optiprep Gradient

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	

3. Balance two tubes against each other.
4. Centrifuge 4 hours at 150,000 × *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 × *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 µl per test with 20-fold dilution
   b. Acid phosphatase activity: 40–80 µl per test without dilution
   c. β-N-Acetylglucosaminidase activity: For cells 10 µl per test without dilution; for tissues 2.5–5 µl per test with 2–4 fold dilution
   d. Cytochrome c oxidase activity: 2.5–25 µl per test with 5-fold dilution
   e. Cytochrome c reductase activity: 25 µl per test without dilution
   f. Catalase activity: for cell extracts 10 µl per test with 2-fold dilution; for tissues 5 µl 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 µl per test with 100-fold dilution; for cell extracts 10–40 µl per test with 10-fold dilution
   b. Acid phosphatase activity: for tissues 5–10 µl per test; for cell extracts 10–40 µl per test, both without dilution
   c. β-N-Acetylglucosaminidase activity: For tissues 10–20 µl per test; for cells 10 µl per test, both without dilution
   d. Cytochrome c oxidase activity: for tissues 5–10 µl per test with 20-fold dilution; for cell extracts 50 µl per test with 2-fold dilution
   e. Cytochrome c reductase activity: for tissues 2.5–5 µl per test; for cell extracts 50 µl per test, both without dilution
   f. Catalase activity: for cell extracts 10 µl per test with 2–3 fold dilution; for tissues 2.5–5 µl 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.

This procedure will result in the separation of the organelles (see Figure 1).

Figure 1.
Separation of Lysosomes from Rat Liver on an Optiprep Gradient



8 mg of protein of the CLF were used to prepare the DOF and then placed on a 5 ml step gradient (step II, A, 2). The blue arrows show the approximate position of the Optiprep gradient.

Note: Alternative preparation of DOF - In order to achieve good separation of the lysosomes, it is possible to change the initial OptiPrep Density Gradient Medium concentration in the sample. This may lead to slightly different contamination with other organelles. The preparation of a sample (DOF) with a 16% OptiPrep Density Gradient Medium concentration, a recommended alternative, is shown in Table 4.

Table 4.
Preparation of 16% Diluted Optiprep Fraction (DOF) for Loading on the Gradient

Sample	Optiprep (D1556) (ml)	Optiprep Dilution Buffer (ml)	2.3 M Sucrose (ml)	CLF (ml)	mOsm
Liver	0.425	0.72	0.04	0.4	292

Option B: Further purification of density gradient fractions with the aid of calcium chloride

1. Prepare a 250 mM Calcium Chloride Solution as described under Preparation Instructions.
2. Take a 200 µl aliquot of each fraction from the Optiprep gradient and add the 250 mM Calcium Chloride Solution to a final concentration of 8 mM (6.5 µl per 200 µl of sample). Mix.
3. Incubate on ice for 15 minutes and then centrifuge at 5,000 × *g* for 15 minutes in a microcentrifuge.
4. Transfer the supernatant liquid into a fresh tube. The majority of the lysosomes (>50%) will remain in the supernatant.

Option C: Direct addition of calcium chloride to the DOF

If no ultracentrifuge is available and there is only a limited amount of material, it is possible to remove contaminating mitochondria and endoplasmic reticulum by direct addition of the 250 mM Calcium Chloride Solution (to a final concentration of 8 mM) to the sample prepared for the Optiprep gradient (DOF, see Table 2). That will give a lower yield of purified lysosomes than the gradient purification (25% rather than >50%), but is a much simpler procedure

III. Storage of crude and purified lysosomes

The CLF may be kept overnight at 2–8 °C and then separated the next day on a density gradient. Also, the purified lysosomes may be kept for up to 24 hours with little degradation. If a Western blot of specific proteins in the lysosome is desired, it is advisable to add the sample buffer immediately after preparation of the purified fraction and then to freeze the sample.

IV. Measurement of intactness of the Lysosomes

The intactness of the lysosomes can be assessed using the dye Neutral Red. The absorbance maximum of the Neutral Red dye shifts from 460 nm to 510 nm in the acidic pH conditions found in the lysosome. The Neutral Red dye will concentrate in the lysosomes, where it binds by electrostatic hydrophobic bonds with anionic sites in the lysosomal matrix.[9,10] The uptake may be followed by a spectrophotometer in real time.

1. Set up a spectrophotometer on “Time Drive” using a program for 3 minutes at the two wavelengths 460 and 510 nm.
2. Place up to 990 µl of 1× Extraction Buffer in a 1 ml disposable cuvette. The volume is dependent on the amount of sample to be added to the cuvette.
3. Add 2.5 µl of Neutral Red Reagent (Catalog Number N2537) and mix well by inversion.
4. Add the requisite amount of lysosomes (30–120 µg-protein, Options A, B, or C) up to 100 µl volume and start the program.
5. Obtain the values for each wavelength in the time period from 5–65 seconds.

Note: If a spectrometer is not available with a time drive option and multiple wavelength readings, the same sample can be measured at 460 nm and 510 nm consecutively.

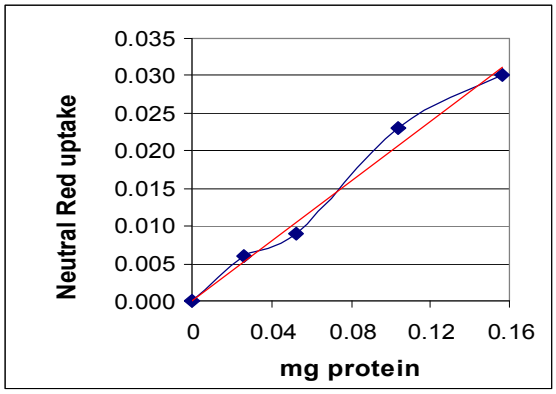
Calculation

Since the dye absorbance at 460 nm changes very little during uptake, any change in this value is probably a result of fluctuations due to the biological material added. This effect can be removed by subtracting the value obtained at 460 nm from that obtained at 510 nm. The value desired is:

Neutral Red Uptake = $\Delta A_{(510)}$/min – $\Delta A_{(460)}$/min

An example of the linearity of the dye uptake with increasing amounts of lysosomal protein is shown in Figure 2.

Figure 2.
Linearity of Dye Uptake with Increasing Amounts of Lysosomal Protein



The suspended 20,000 × *g* pellet (step I, A, 10 or I, B, 11) was added to the mixture at varying amounts of protein. The uptake was linear from 25–150 μg of protein.

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Appendix 1
Flowchart of Lysosome Preparation