

Lysosome Enrichment Kit for Tissue and Cultured Cells

89839

1845.0

Number	Description
89839	Lysosome Enrichment Kit for Tissue and Cultured Cells , contains sufficient reagents for 25 enrichments of intact lysosomes from tissue and cultured cells Kit Contents: Lysosome Enrichment Reagent A , 90 ml Lysosome Enrichment Reagent B , 90 ml OptiPrep™ Cell Separation Media , 50 ml BupH™ Phosphate Buffered Saline , 1 pack, results in 0.1 M sodium phosphate, 0.15 M sodium chloride; pH 7.2 when constituted with 500 ml of ultrapure water Storage: Upon receipt store at 4°C. Product shipped at ambient temperature.

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Introduction

The Lysosome Enrichment Kit for Tissue and Cultured Cells enables isolation and enrichment for intact lysosomes from crude cell and tissue extracts. The kit provides sufficient reagents for preparing 25 extracts and uses OptiPrep™ Cell Separation Media for the density-based separation of lysosomes from contaminating cell structures. The isolated lysosomes may be used for a number of downstream applications, including 2D/MS for proteomics research, electron microscopy, disease profiling and gene expression, signal transduction, and interaction or localization.

Important Product Information

- The OptiPrep™ Cell Separation Media is a 60% solution of iodixanol – 5,5'-[(2-hydroxy-1,3-propanediyl)-bis(acetylamino)] bis [*N,N'*-bis(2,3 dihydroxypropyl-2,4,6-triiodo-1,3-benzenecarboxamide)].
- The density of the lysosomes varies depending on the cell and tissue source. Therefore, optimization of the gradient concentrations is necessary for best results. Refer to the Axis-Shield website for helpful density gradient concentrations for various cell and tissue types: www.axis-shield.com
- The procedure was developed using a tabletop ultracentrifuge. A floor centrifuge may also be used with appropriate scaling of reagents.

Additional Materials Required

- Ultracentrifuge: tabletop or floor-model
- Ultracentrifuge tubes: either open- or closed-top tubes, for example, 8 ml
- Bench-top microcentrifuge with refrigeration
- 2 ml microcentrifuge tubes
- 15 ml conical tubes
- Vortex mixer
- Protease inhibitors, such as Halt™ Protease Inhibitor Cocktail Kit, EDTA-Free (Product No. 78415)
- Dounce tissue grinder, such as 2 ml Kontes or Wheaton Dounce Tissue
- Tissue Tearer, such as the Kinematica AG Polytron® PT1200
- Sonicator, such as the Misonix Sonicator 3000
- Surgical scissors for mincing tissue

Procedure

A. Material Preparation

Phosphate-buffered Saline (PBS)	Dissolve the dry-blend buffer with 500 ml of ultrapure water. For long-term storage of excess buffer, sterile-filter the solution and store at 4°C.
Gradient Dilution Buffer	Mix equal volumes of the Lysosome Enrichment Reagents A and B. Each sample requires 2.5 ml of Gradient Dilution Buffer.
OptiPrep™ Gradients	The OptiPrep™ Media is supplied as a 60% solution. Prepare five gradient solutions using the Gradient Dilution Buffer as indicated in the table below. Each volume is for one sample preparation.

Mix		Final Volume (μ l)	Gradient %
OptiPrep™ Media Volume (μ l)	Gradient Dilution Buffer Volume (μ l)		
283.3	716.7	1,000	17
333.3	666.7	1,000	20
191.7	308.3	500	23
450	550	1,000	27
250.0	250.0	500	30

B. Preparation of Cell or Tissue Extracts

Protocol 1: Cultured Cells and Dounce Homogenization

- Immediately before use, add protease inhibitors to Lysosome Enrichment Reagents A and B; only add inhibitors to the volume being used for the procedure and not to the stock solutions.
- Process one sample at a time.
- Pre-chill Dounce tissue grinder on ice before use.

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- Empirically determine the number of Dounce strokes required for optimal cell lysis with minimal damage to the lysosomes for each tissue type. See the Additional Information Section for cell lysis information.
1. Pellet 50-200 mg of cells by centrifuging harvested cell suspension in a 2.0 ml microcentrifuge tube at $\sim 850 \times g$ for 2 minutes. Carefully remove and discard the supernatant.
 2. Add 800 μ l of Lysosome Enrichment Reagent A. Vortex at medium speed for 5 seconds and incubate on ice for exactly 2 minutes. Do not exceed the 2 minute incubation.
 3. Transfer cell suspension to a Dounce tissue grinder and homogenize cells on ice. Perform enough strokes to effectively lyse the cells.
Note: To check lysis efficiency, place 5 μ l of lysate onto a glass slide, add coverslip and view with a microscope. Compare results with 5 μ l of the non-lysed cells.
 4. Transfer lysed cells into a 2 ml microcentrifuge tube and add 800 μ l of Lysosome Enrichment Reagent B. Invert tube several times to mix – do not vortex.
 5. Centrifuge tube at $500 \times g$ for 10 minutes at 4°C. Collect supernatant in a new tube and keep on ice until needed.
 6. Proceed to Sections C and D for gradient centrifugation and isolation of lysosomes.

Protocol 2: Cultured Cells and Sonication

- Immediately before use, add protease inhibitors to Lysosome Enrichment Reagents A and B; only add inhibitors to the reagent amount being used for the procedure and not to the stock solutions.
1. Pellet 50-200 mg of cells by centrifuging harvested cell suspension in a 2 ml microcentrifuge tube at $\sim 850 \times g$ for 2 minutes. Carefully remove and discard the supernatant.
 2. Add 800 μ l of Lysosome Enrichment Reagent A. Vortex at medium speed for 5 seconds and incubate on ice for exactly 2 minutes. Do not exceed the 2 minute incubation.
 3. Sonicate cell suspension on ice, performing a sufficient number of bursts for effective cell lysis (e.g. 10-15 burst, at 6-9W of power).
Note: To check the lysis efficiency, place 5 μ l of cell lysate onto a glass slide, add coverslip and view under a microscope. Compare with 5 μ l of non-lysed cells.
 4. Add 800 μ l of Lysosome Enrichment Reagent B. Invert tube several times to mix – do not vortex.
 5. Centrifuge tube at $500 \times g$ for 10 minutes at 4°C. Collect supernatant in a new tube and keep on ice until needed.
 6. Proceed to Sections C and D for gradient centrifugation and isolation of lysosomes.

Protocol 3: Soft Tissue and Dounce Homogenization

- Pre-chill the Dounce tissue grinder on ice before use.
 - Immediately before use, add protease inhibitors to the Lysosome Enrichment Reagents A and B; add inhibitors only to the volume being used for the procedure and not to the stock solutions.
 - Empirically determine the number of Dounce strokes required for optimal cell lysis with minimal damage to the lysosomes for each tissue type. See Additional Information Section for cell lysis information.
1. Wash 50-200 mg of tissue with 2-4 ml of PBS. Carefully remove and discard the PBS wash.
 2. Mince tissue into small pieces (less than 3 mm³) and add 800 μ l of Lysosome Enrichment Reagent A.
 3. Perform Dounce homogenization on ice.
Note: To check lysis efficiency, place 5 μ l of lysate onto a glass slide, add coverslip and view with a microscope. Compare results with 5 μ l of the non-lysed cells.
 4. Add 800 μ l of Lysosome Enrichment Reagent B. Invert the tube several times to mix – do not vortex.
 5. Centrifuge tube at $500 \times g$ for 10 minutes at 4°C. Collect supernatant in a new tube and keep on ice until needed.
 6. Proceed to Sections C and D for gradient centrifugation and isolation of lysosomes.

Protocol 4: Soft Tissue and the Polytron® Tissue Tearer

- Immediately before use, add protease inhibitors to the Lysosome Enrichment Reagents A and B; add inhibitors only to the reagent amount being used for the procedure and not to the stock solutions.
 - The speed and time duration for homogenization varies depending on tissue type.
1. Wash 50-200 mg of tissue with 2-4 ml of PBS. Carefully remove and discard the PBS wash.
 2. Mince tissue into small pieces (less than 3 mm³) and add 800 µl of Lysosome Enrichment Reagent A.
 3. Homogenize on ice at approximately 8,000-9,000 rpm for 45 seconds; however, depending on tissue type, the time required for lysis may be longer or shorter.

Note: To check lysis efficiency, place 5 µl of lysate onto a glass slide, add coverslip and view with a microscope. Compare results with 5 µl of the non-lysed cells.

4. Add 800 µl of Lysosome Enrichment Reagent B. Invert the tube several times to mix – do not vortex.
5. Centrifuge at 500 × g for 10 minutes at 4°C. Collect supernatant in a new tube and keep on ice until needed.
6. Proceed to Sections C and D for gradient centrifugation and isolation of lysosomes.

Protocol 5: Hard Tissue and Dounce Homogenization

- Pre-chill the Dounce tissue grinder on ice before use.
 - Immediately before use, add protease inhibitors to the Lysosome Enrichment Reagents A and B; add inhibitors only to the reagent amount being used for the procedure and not to the stock solutions.
 - Empirically determine the number of Dounce strokes required for optimal cell lysis with minimal damage to the lysosomes for each tissue type. See the Additional Information Section for cell lysis information.
1. Wash 50-200 mg of tissue with 2-4 ml of PBS. Carefully remove and discard the PBS wash.
 2. Mince tissue into small pieces (less than 3 mm³) and add 800 µl of Lysosome Enrichment Reagent A.
 3. Perform Dounce homogenization on ice.

Note: To check lysis efficiency, place 5 µl of lysate onto a glass slide, add coverslip and view with a microscope. Compare results with 5 µl of the non-lysed cells.

4. Add 800 µl of Lysosome Enrichment Reagent B. Invert the tube several times to mix – do not vortex.
5. Centrifuge at 500 × g for 10 minutes at 4°C. Collect supernatant in a new tube and keep on ice until needed.
6. Proceed to Sections C and D for gradient centrifugation and isolation of lysosomes.

Protocol 6: Hard Tissue and the Polytron® Tissue Tearer

- Immediately before use, add protease inhibitors to the Lysosome Enrichment Reagents A and B; add inhibitors only to the reagent amount being used for the procedure and not to the stock solutions.
 - The speed and time duration for homogenization varies depending on cell/tissue type.
1. Wash 50-200 mg of tissue with 2-4 ml of PBS. Carefully remove and discard the PBS wash.
 2. Mince tissue into small pieces (< 3 mm³) and add 800 µl of Lysosome Enrichment Reagent A.
 3. Homogenize on ice at approximately 8,000-9,000 rpm for 45 seconds; however, depending on tissue type, the time required for lysis may be longer or shorter.

Note: To check lysis efficiency, place 5 µl of lysate onto a glass slide, add coverslip and view with a microscope. Compare results with 5 µl of the non-lysed cells.

4. Add 800 µl of Lysosome Enrichment Reagent B. Invert the tube several times to mix – do not vortex.
5. Centrifuge at 500 × g for 10 minutes at 4°C. Collect supernatant in a new tube and keep on ice until needed.
6. Proceed to Sections C and D for gradient centrifugation and isolation of lysosomes.

C. Preparation of the Sample for Density Gradient Centrifugation

- The concentrations of the density media for lysosome isolation may require optimization, depending on the cell or tissue type. Refer to references for examples of enrichment gradient concentrations and cell/tissue sources.
 - Use either an open- or closed-top ultracentrifuge tube for enrichment.
1. In an ultracentrifuge tube, prepare a discontinuous density gradient by carefully overlaying the prepared OptiPrep™ Gradients (see Section A) in descending concentrations. For example, first add the 30% gradient and then the 27%, 23%, 20% and 17% gradients.
 2. Mix the prepared cell or tissue extract (Section B) with the OptiPrep™ Cell Separation Media to make a final concentration of 15% OptiPrep™ Media. For example, add 1,500 µl of extract to 500 µl of OptiPrep™ Media.
 3. Overlay the sample containing the 15% OptiPrep™ Media on top of the density gradients (Figure 1).
 4. Ultracentrifuge the samples at 145,000 × g for 2 hours at 4°C.
 5. After centrifugation, several bands will form in the gradient (Figure 2). The lysosome band is located in the top 2 ml of the gradient. Carefully remove the band and save on ice.

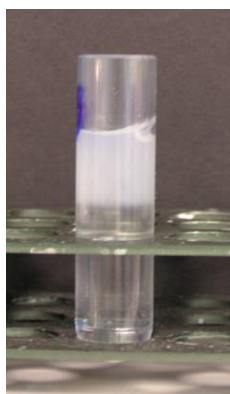


Figure 1. Before ultracentrifugation, the sample is visible on top of the gradient. The sample is A431 cell extract prepared from ~200 mg of cell pellet.

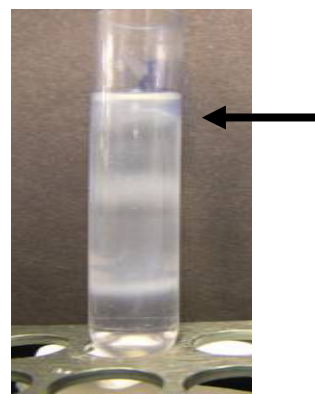


Figure 2. After ultracentrifugation, bands form in the gradient. The top band contains the isolated lysosomes.

D. Removal of Lysosomes from OptiPrep™ Media

1. Mix the isolated lysosome fraction with 2-3 volumes of PBS to decrease the concentration of the OptiPrep™ Media. Gently vortex to mix the sample.
2. Transfer the sample into a microcentrifuge tube and centrifuge at 18,000 × g for 30 minutes at 4°C.
3. Remove the supernatant and keep the lysosome pellet on ice.
4. To surface-wash the pellet, add 1 ml of the Gradient Dilution Buffer and centrifuge at 18,000 × g for 30 minutes at 4°C.
5. Remove supernatant and maintain the lysosome pellet on ice until downstream processing. Freezing and thawing may compromise lysosomal integrity.

Troubleshooting

Problem	Possible Cause	Solution
Small lysosome pellet	Number of Dounce strokes were insufficient to achieve optimal lysis	Optimize the number of Dounce strokes for each tissue type
	Cells are difficult to lyse	Increase the number of Dounce strokes and consider using the Sonicator and Polytron Tissue Tearer
	Hard-tissue sample is not completely homogenized	Mince hard tissue into small pieces before homogenization
Fats/lipids are present in the sample	Dietary intake contributes to fats/lipids in the system	Starve animal 24 hours before sacrifice to minimize the amount of fats/lipids present in the system
	Rats are mature or overweight	Young lean rats have less fats/lipids in their systems
Lysosomes are in several fractions or dispersed in the gradient	Lysosome density differs depending on the cell or tissue type	Optimize the gradient concentrations for each cell or tissue type
	Inadvertent mixing of gradients during sample removal from the gradient	Siphon lysosome band or other fraction carefully and slowly – quickly removing the sample causes mixing
Lysosomes are not completely pelleted	The density gradient concentration is not low enough	Dilute the gradient concentration with PBS to pellet the lysosomes

Additional Information

A. Cell Lysis Information

Note: Regardless of the cell lysis method, monitor the lysis efficiency during optimization. Do not jeopardize organelle integrity by significantly increasing cell lysis efficiency.

Dounce Homogenization: The number of Dounce homogenization strokes necessary for optimal lysis varies depending upon cell or tissue type. For each sample type, determine the number of strokes required for optimal lysis with minimal damage to the lysosomes. Use the table below as a general guide.

<u>Tissue Type</u>	<u>Tissue*</u>		<u>Cell Type</u>	<u>Cells**</u>	
	<u>Amount (mg)</u>	<u>Number of Strokes</u>		<u>Amount (mg)</u>	<u>Number of Strokes</u>
Soft (Liver)	50	20-30	A431	50	30-50
	200	50-75		200	80-100
Hard (Heart)	50	10	HeLa	50	20-30
	200	30-50		200	70-80

*Mince the tissue into small pieces before homogenization.

** Determine lysis efficiency by visual examination with a microscope.

Sonication: The robustness of the cell type affects the power output and the number of bursts needed for effective cell lysis. The procedure detailed here was tested on HeLa, HepG2 and A431 cells and used 9W of power and 15 sonication bursts. During optimization, monitor lysis efficiency by spotting sample unto a glass slide and comparing with a whole cell control.

Polytron® Tissue Tearer: Depending on the tissue type, adjust speed and duration as needed. For soft tissue, such as liver, and hard tissue, such as heart and kidney, the protocol suggests using 8,000-9,000 rpm for 45 seconds. The speed and duration required also varies depending on the amount of starting tissue material. During optimization, monitor the lysis efficiency by placing sample onto a glass slide and comparing with a non-lysed control.

B. Optimization of OptiPrep™ Cell Separation Media Concentration and Centrifugation Speed/time

Depending on the cell or tissue type, lysosomes differ in morphology and relative density. Lysosomes vary from 0.2 to 2 μm and can fuse with other lysosomes or endosomes. Organellar fusion affects relative densities of the organelle and directly affects the concentration of OptiPrep™ Media required for an effective enrichment. Therefore, the optimum gradient concentration must be determined empirically. See references for examples of various OptiPrep™ Media concentrations with different cell and tissue types. In the initial experiment, collect small volumes of the sample/gradient, for example 500-1,000 μl (post-centrifugation), from the top of the gradient going down. Analyze the fractions by Western blot to determine the location of the lysosomes and the contaminating organelles to help determine a good starting concentration to use. Centrifugation speed and time also may require optimization. Depending on the sample, it may be necessary to centrifuge longer or faster. Perform the developed protocol initially and make the necessary optimizations afterwards.

C. Lysosome Lysis

For protein analysis using the Coomassie Plus – The Better Bradford™ Assay Kit (Product No. 23236), the lysosomes may be lysed with 2% CHAPS in Tris-buffered saline (TBS; 25 mM Tris, 0.15 M NaCl; pH 7.2; Product No. 28379) as follows:

- For direct analysis by SDS-PAGE or Western blotting, boil lysosomes pellet with SDS-PAGE sample buffer and apply to a protein electrophoresis gel.
 - Depending on the size of the lysosome pellet or protein concentration needed, add 100-400 µl of 2% CHAPS in TBS to the lysosome pellet and vortex for 1 minute.
1. Incubate at room temperature for 10 minutes.
 2. Vortex sample for 1 minute.
 3. Centrifuge sample at 18,000 × g at 4°C for 5 minutes. The clarified supernatant will contain soluble lysosomal proteins that can be analyzed by Coomassie Plus – the Better Bradford™ Assay Kit (Product No. 23236).

Related Pierce Products

89840	Peroxisome Enrichment Kit for Tissue
89841	Nuclei Enrichment Kit for Tissue
78833	NE-PER® Nuclear and Cytoplasmic Extraction Kit
89826	Mem-PER® Eukaryotic Membrane Protein Extraction Kit
78415	Halt™ Protease Inhibitor Cocktail, EDTA-Free, 1 ml
23236	Coomassie Plus – The Better Bradford™ Assay Kit
78501	M-PER® Mammalian Protein Extraction Reagent, 250 ml
34095	SuperSignal® West Femto Maximum Sensitivity Chemiluminescent Substrate, 100 ml

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Current versions of product instructions are available at www.piercenet.com. For a faxed copy, call 800-874-3723 or contact your local distributor.

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