Lysosome Enrichment Kit for Tissue and Cultured Cells

89839

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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.

Warranty: Pierce Biotechnology (hereafter "Pierce") products are warranted to meet stated product specifications and to conform to label descriptions when stored and used properly. Unless otherwise stated, this warranty is limited to one year from date of sale when used according to product instructions. Pierce’s sole liability for the product is limited to replacement of the product or refund of the purchase price. Unless otherwise expressly authorized in writing by Pierce, products are supplied for research use only and are intended to be used by a technically qualified individual. Pierce’s quality system is certified to ISO 9001. Pierce makes no claim of suitability for use in applications regulated by FDA. Pierce strives for 100% customer satisfaction. If you are not satisfied with the performance of a Pierce product, please contact Pierce or your local distributor.
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.

<table>
<thead>
<tr>
<th>Mix</th>
<th>OptiPrep™ Media Volume (µl)</th>
<th>Gradient Dilution Buffer Volume (µl)</th>
<th>Final Volume (µl)</th>
<th>Gradient</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>283.3</td>
<td>716.7</td>
<td>1,000</td>
<td>17</td>
</tr>
<tr>
<td></td>
<td>333.3</td>
<td>666.7</td>
<td>1,000</td>
<td>20</td>
</tr>
<tr>
<td></td>
<td>191.7</td>
<td>308.3</td>
<td>500</td>
<td>23</td>
</tr>
<tr>
<td></td>
<td>450</td>
<td>550</td>
<td>1,000</td>
<td>27</td>
</tr>
<tr>
<td></td>
<td>250.0</td>
<td>250.0</td>
<td>500</td>
<td>30</td>
</tr>
</tbody>
</table>

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.
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 ~850 × g for 2 minutes. Carefully remove and discard the supernatant.
2. Add 800 µl 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 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. Transfer lysed cells into a 2 ml microcentrifuge tube and add 800 µl of Lysosome Enrichment Reagent B. Invert tube several times to mix – do not vortex.
5. Centrifuge tube 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 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 ~850 × g for 2 minutes. Carefully remove and discard the supernatant.
  2. Add 800 µl 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 µl of cell lysate onto a glass slide, add coverslip and view under a microscope. Compare with 5 µl of non-lysed cells.
  4. Add 800 µl of Lysosome Enrichment Reagent B. Invert tube several times to mix – do not vortex.
  5. Centrifuge tube 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 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 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 tube 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 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 \( \times \) 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.

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 \( \times \) 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 \( \times \) 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**

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

**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.

<table>
<thead>
<tr>
<th>Tissue Type</th>
<th>Amount (mg)</th>
<th>Number of Strokes</th>
<th>Cell Type</th>
<th>Amount (mg)</th>
<th>Number of Strokes</th>
</tr>
</thead>
<tbody>
<tr>
<td>Soft (Liver)</td>
<td>50</td>
<td>20-30</td>
<td>A431</td>
<td>50</td>
<td>30-50</td>
</tr>
<tr>
<td></td>
<td>200</td>
<td>50-75</td>
<td>HeLa</td>
<td>200</td>
<td>80-100</td>
</tr>
<tr>
<td>Hard (Heart)</td>
<td>50</td>
<td>10</td>
<td></td>
<td>50</td>
<td>20-30</td>
</tr>
<tr>
<td></td>
<td>200</td>
<td>30-50</td>
<td></td>
<td>200</td>
<td>70-80</td>
</tr>
</tbody>
</table>

*Mince the tissue into small pieces before homogenization.

**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. Organelar 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 lysosomal 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

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


