Minute™ Endosome Isolation and Cell Fractionation Kit

Cat. No. ED-028

Description

Early endosomes (EE) provide the starting point for late endosome maturation. the EEs are mainly derived from primary endocytic vesicles that fuse with each other. EEs receive endocytic cargo not only through the clathrin-mediated pathway but several other pathways. In addition to their roles in normal cell physiology, endocytic processes play a key role in many diseases such as Alzheimer's disease and inherited lysosomal storage diseases. Traditional methods for isolating endosomes are based on density gradient ultracentrifugation. The protocol requires large amount of starting material and the methods are tedious and time consuming. The minute endosome isolation kit employees a patented spin-column based novel technology for endosome isolation, which is rapid, simple and requires smaller number of cultured cells or milligram amounts of tissues. This kit can precipitate and significantly enrich early endosomes from cultured cells or tissues. The availability of the kit should facilitate the research in the field.

Kit Components

Buffer A	15 ml
Buffer B	15 ml
Plastic rods	2
Filter Cartridge	20
Collection Tube	20
Tissue dissociation beads	2.5g

Additional Materials Required

1 X PBS, Vortexer, Table-Top Micro centrifuge

Shipping and Storage: Ship at ambient temperature and store the kit at 4°C

Important Information:

1. Read the entire procedures carefully. Chill protein extraction filter cartridge with collection tube on ice prior to use.
2. All centrifugation steps should be performed at 4°C in a cold room or in a refrigerated microfuge.
3. To study protein phosphorylation, **phosphatase inhibitors** (such as PhosStop from Roche) should be added to buffer A prior to use. The use of protease inhibitor cocktails is optional.
4. It is recommended to use BCA Protein Assay Kit for determination of protein concentration (Pierce, Cat #:23227).

Protocol

1. Place the filter cartridges in collection tubs, and incubate on ice.

2. **For cultured cells**, collect 10-30 X 10^6 cells by low speed centrifugation (500-600 X g for 5 min). Go to step 3a. **For tissue samples**, go to step 3b.

3a. Wash cells once with cold PBS. Remove supernatant completely and resuspend the pellet in 500 µl buffer A. Incubate the cell suspension on ice for 5-10 min. **Vortex the tube vigorously for 10-30 seconds**. Immediately transfer the cell suspension to the filter cartridge. Go to step 4.

3b **For tissue samples,** place 10-20 mg tissue (fresh or frozen) or frozen in a filter cartridge. Add 200 µl buffer A to the filter and grind the tissue with a plastic rod for one min by pushing the tissue against the surface of the filter repeatedly with twisting force (Note: if you are working with skeletal or cardiac muscles, it is recommended to add about 100 mg tissue dissociation beads to the filter prior to grinding). Add 300 µl buffer A to the same filter cartridge, mix by pipette up and down a few times and incubate the tube on ice with **cap open** for 5 min. Go to step 4.

Note: The presence of some un-homogenized tissue will not affect the quality of the sample. The plastic rod is reusable. Clean it with 70% alcohol or water.

4. Cap the filter cartridge and centrifuge at 16,000 X g for 30 seconds **(it is recommended to use a table top centrifuge that can reach maximum speed in less than 10 seconds**). The flow through in the collection tube can be resuspended and re-pass through the same filter again. This may increase the final endosome yield.

5. Discard the filter and resuspend the pellet by vigorously vortexing for 10 seconds. Centrifuge at 3000 rpm (700 X g) for 2-3 min (the pellet contains intact nuclei and some un-ruptured cells).

6. Transfer the supernatant to a fresh 1.5 ml microfuge tube and centrifuged at 4°C for 30-60 min at 16,000 X g (longer centrifugation time can increase purity). After centrifugation, transfer the supernatant to a fresh 1.5 ml tube. The pellet contains mainly larger organelles and plasma membranes.

7. Measure the volume of the supernatant from step 6 after 16,000 X g spinning and mix with ½ of buffer B by vortexing briefly (buffer B to supernatant ratio: 1:2). Incubate the tube at 4°C for 1h to overnight (longer incubation can increase yield). The buffer B to the supernatant ratio can be reduced or increased from 0.25:1 to 1:1 depending on the final yield of endosomes.

8. Centrifuge at 10,000 X g for 30 min at 4°C. Remove the supernatant and save it if desired (this is cytosolic fraction). The pellet contains isolated endosomes. The yield is typically 20-100 µg/sample. The pellet can be resuspended in any buffer of your choice but following reagents are recommended depending upon downstream applications.

Tech Notes:

1. The yield and purity of isolated endosomes may vary depending upon specific tissue/cell types.
2. If there is no pellet in step 8 after 10,000 X g spin, double the starting material and pass cells through the filter in step 4 twice.
3. The buffer B to supernatant ratio can be adjusted according to the final results. Increase the ratio to 1:1 if the yield is low, decease the ratio if excessive cytosolic protein contamination is an issue.
4. To assess the yield and purity of the isolated endosomes, we recommend comparing them to the total cell/tissue lysate in Western blotting (WB) using an antibody specific to endosomes such as EEA1. It's crucial to ensure equal protein loading in SDS-PAGE. Staining the post-transfer blot with Ponceau Red can provide insights into significant variations in protein loading.
5. The isolated endosome pellet is water-insoluble and must be dissolved in a detergent-containing buffer for protein quantification. If the pellet doesn't solubilize efficiently using WA-009 (see table below), add SDS to WA-009 to reach a final concentration of 0.4% and increase the volume of the protein solubilization reagent. Some components in buffer B might interfere with mass spectrometry analysis and should be removed after trypsin digestion.
6. Buffer B contains PEG that may interfere with mass spectrometry and needs to be removed prior to the analysis (reference: Zhao C, O'Connor PB. Removal of polyethylene glycols from protein samples using titanium dioxide. Anal Biochem. 2007 Jun 15;365(2):283-5).

Following protein solubilization reagents are recommended.

Product Name	Cat. No.	Applications
Minute™ Denaturing Protein Solubilization Reagent	WA-009	SDS-PAGE electrophoresis and Western blotting, trypsin digestion, purification of proteins with biotin labeling or histidine labeling, etc.
Minute™ Non-Denatured Protein Solubilization Reagent	WA-010	ELISA, immunoprecipitation/Co-IP, enzymatic activity determination and other applications.
Minute™ Protein Solubilization Reagent for MS	WA-011	Trypsin digestion and subsequent mass spectrometry analysis.