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Minute™ ER Enrichment Kit

(For Tissues and Cultured Cells)

Cat. No. ER-036

Description

The endoplasmic reticulum (ER) is a significant membranous structure that bridges the nuclear membrane and the plasma membrane, serving as a pivotal player in the exocytic pathway of protein trafficking in all eukaryotic cells. Proteins synthesized in the cytoplasm are directed to the ER, from where vesicles transport protein cargo to the Golgi apparatus, leading to subsequent fusion with the plasma membrane.

Traditional ER isolation methods rely on density gradient ultracentrifugation, a process demanding a substantial amount of starting material. These methods can be laborious, time-consuming, and often result in notable cross-contamination. Remarkably, all existing commercial kits for ER isolation are based on techniques developed in the 1970s. In a departure from these traditional approaches, the Minute™ ER enrichment kit stands out in the market by harnessing patented spin-column-based technology. This innovative method is not only straightforward and rapid but also requires only a small quantity of starting cultured cells or tissues.

This kit excels at differentially precipitating native ER, primarily rough ER, from cultured cells and tissues, all without the need for a Dounce homogenizer or ultracentrifugation. The entire protocol can be completed in approximately two hours.

Kit Components (20 prep)

1.	Buffer A	20 ml
2.	Buffer B	1 ml
3.	Buffer C	1 ml
4.	Buffer D	10 ml
5.	Plastic rods	2
6.	Filter Cartridge	20
7.	Collection Tube	20

Additional Materials Required

1 X PBS, Vortexer, Table-Top Micro centrifuge with maximum speed of 16,000 X g.
(The centrifuge should be able to reach maximum speed within 10 seconds)

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

Important Information:

1. Carefully review the entire procedure, and before use, ensure the filter cartridge with the collection tube is chilled on ice.
2. All centrifugation steps must be carried out at 4°C, either in a cold room or using a refrigerated microcentrifuge.

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3. For investigations involving protein phosphorylation, it is essential to incorporate phosphatase inhibitors (such as PhosStop from Roche) into buffer A before usage. If concerns about protein degradation arise, include protease inhibitor cocktails in buffer A prior to use.
4. We recommend employing the BCA Protein Assay Kit for determining protein concentration (Pierce, Cat #:23227).
5. Be aware that the yield and purity of the isolated ER may fluctuate based on specific cell/tissue types and the quantity of starting material used. Optimizing the protocol may be necessary to achieve the best results (see technical notes below).

Protocol

Note: Warm buffer D to room temperature and mix well prior to use.

1. Begin by placing the filter cartridges in a collection tube and incubating them on ice.
2. **For cultured cells:** collect 25-35 X 10^6 cells through low-speed centrifugation (500-600 X g for 5 min). Wash the cells once with cold PBS, ensuring the complete removal of the supernatant. Freeze the pellet at -70-80°C for 10 minutes. Resuspend the pellet in 550 µl of buffer A. Vigorously vortex the tube for 20-30 seconds, and promptly transfer the cell suspension to a filter cartridge. Proceed to step 3.
   For tissue samples: place 30-40 mg of frozen tissue (fresh tissue should be frozen at -20 or -80°C for at least overnight) in a filter cartridge. Add 200 µl of buffer A to the filter and grind the tissue with a reusable plastic rod for 2-3 minutes by repeatedly pushing the tissue against the filter's surface with a twisting force. After grinding, add 350 µl of buffer A to the same filter cartridge, mix by pipetting up and down a few times, and move on to step 3. The plastic rod can be cleaned with 70% alcohol or water.
3. Cap the filter cartridge, invert the tube a few times, and then centrifuge at 16,000 X g for 30 seconds. ***(Optional: The flow-through in the collection tube can be resuspended and passed through the same filter again to increase the yield.)***
4. Discard the filter and resuspend the pellet by vigorously vortexing for 10 seconds. Centrifuge at 2,000 X g for 3 minutes. The pellet contains nuclei, large cell debris, and some un-ruptured cells.
5. Transfer all the supernatant to a fresh 1.5 ml microfuge tube, keeping in mind to minimize the inclusion of lipids, especially for liver tissue. Centrifuge at 4°C at 8,000 X g for 10 minutes. After centrifugation, carefully transfer 400 µl of the supernatant to a fresh 1.5 ml tube, taking care to avoid any lipids that may be present. The pellet primarily contains larger cell debris, mitochondria, lysosomes, and plasma membranes.
6. Add 40 µl of buffer B to the 400 µl of supernatant. Mix thoroughly by brief vortexing (the ratio of buffer B to supernatant is 1:10). Incubate the tube at 4°C for 20-30 minutes.
7. Centrifuge at 8,000 X g for 10 minutes. Completely remove the supernatant and resuspend the pellet in 400 µl of cold buffer A by pipetting up and down 40-50 times, followed by vigorous vortexing for 20 seconds (be sure to wash the tube's wall). Add 40 µl of buffer C (1/10 of the resuspended volume) to the tube and vortex briefly. Incubate at room temperature for 10-15 minutes (vortex the tube every five minutes). Centrifuge at 8,000 X g for 5 minutes. Transfer 400 µl of the supernatant to a fresh 1.5 ml tube. Add 400 µl of buffer D to the supernatant and mix briefly by vortexing (the ratio of supernatant to buffer D is 1:1). Incubate the tube at 4°C for 20 minutes.
8. Centrifuge at 10,000 X g for 10 minutes. Remove and discard the supernatant. Briefly spin the tube at 10,000 X g to bring down the residual aqueous phase and remove it completely.
9. Resuspend the pellet in 50 to 200 µl of a detergent-containing buffer. This represents an isolated ER fraction that primarily contains rough ER. If the preparation is not used immediately, add protease inhibitor cocktails to the preparation and store it at -80°C. The content of ER varies significantly in different cell and tissue types. Typically, the isolated ER yield ranges from 20 to 200 µg per sample. The

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water-insoluble ER fraction can be solubilized in any detergent-containing buffer of your choice. However, the reagents listed below are recommended based on your downstream applications.

Tech Notes:

1. In most instances, the crude ER pellet becomes visible following the 8,000 X g centrifugation in step 7. However, for certain cell types, the ER pellet may be less distinct and appear somewhat transparent. If this occurs, you can extend the incubation time in step 6 to 1 hour, and the centrifugation force in step 7 can be increased to 10,000 X g. Even if the pellet is not clearly discernible, it can be assumed that the ER pellet is present, and the protocol should be continued to completion.
2. When cultured cells are utilized, and the final ER yield falls below 20 µg, consider increasing the starting cell count to 50 million. Pellet the cells using low-speed centrifugation as described in step 2. Resuspend the cell pellet in 100 µl of buffer A and freeze it at -20°C for 1 hour. Thaw the cell suspension, pipette it up and down 30-40 times, and transfer the cell suspension to the filter. Homogenize the cell suspension in the filter cartridge by applying the plastic rod against the filter's surface with twisting force around 100 times. Add 0.4 ml of buffer A to the filter cartridge and proceed to step 3.
3. To assess the yield and purity of the isolated ER, we recommend comparing it to total cell/tissue lysate in Western blotting (WB) using an ER-specific antibody, such as calreticulin. Ensuring equal protein loading in SDS-PAGE is of utmost importance. We also suggest staining the post-transfer blot with Ponceau Red to gauge whether there is any notable variation in protein loading.
4. The yield of ER is primarily influenced by two factors: A. the quantity of starting material and B. the efficiency of cell membrane disruption. The efficiency of cell membrane disruption can be easily examined by staining cells with trypan blue before and after passing cells through the filter. Cell viability should be over 90% before and less than 30% after passing through the filter.
5. The extent of ER enrichment varies depending on the sample type. It is recognized that intracellular membranous structures are interconnected, and certain cytosolic marker proteins like actin and tubulin may also associate with organelles. Consequently, it is not unexpected to detect these proteins in the isolated ER fraction.
6. Since the ER and Golgi are physically connected, it is not surprising that Golgi markers are detected in the final ER preparation. The extent of Golgi contamination varies depending on the cell or tissue type, with cell line samples generally being cleaner than tissue samples.
7. If the isolated ER fraction does not exhibit enrichment for ER markers, the following fractions should be subjected to analysis in WB: total cell lysate, supernatants from steps 5 and 7 (after the 8,000 X g spin), the pellet from step 7 following the addition of buffer C and the subsequent spin, and the final isolated ER in step 8. The results obtained from these examinations can provide insights for protocol optimization.

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.