Caveolea&Rafts Isolation Kit

Cat. No. CRI-315K **Lot. No.** (See product label)

SPECIFICATION

Product Overview	The kit provides an easy method for obtaining fractions enriched with caveolea/rafts proteins from tissue culture cells.
Usage	1 Kit is sufficient for 20 purifications
Storage	The Caveolae/Rafts Isolation Kit is shipped on dry ice. The Protease Inhibitor Cocktail and the Anti-Caveolin-1 antibody should be stored at -20 centigrade. It is recommended to store the antibody in working aliquots. After thawing, the aliquot in use can be stored at 2-8 centigrade for at least 4 weeks. The Lysis Buffer and the CTB-HRP should be stored at 2-8 centigrade. The Triton X-100 and the OptiPrep Density Gradient Medium should be stored at room temperature.
Kit Components	• Lysis Buffer 100 ml • Cholera Toxin B Subunit from Vibrio cholerae, Peroxidase Conjugate 50ug • Protease Inhibitor Cocktail for use with mammalian cell and tissue extracts 1ml • Anti-Caveolin-1 antibody produced in rabbit 50ul • Triton X-100 2ml • OptiPrep Density Gradient Medium 100ml
Materials Required but Not Supplied	Ultracentrifuge Rotor TFT 65.13 (Kontron Instruments, fixed angle or equivalent) Cell scraper Ultracentrifuge tubes suitable to the rotor (volume of 10 ml) Horizontal shaker

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	2 ml microcentrifuge tubes Pasteur pipettes, 9 inch Centrifuge tubes, 50 ml (for cells in suspension)
Preparation	It is recommended to use ultrapure water to prepare the reagents. Lysis Buffer containing 1% Triton X-100 Immediately before the assay, prepare 1 ml of lysis buffer containing 1% Triton X-100 for each gradient, by adding 10 ul of Triton X-100 to 1 ml of Lysis Buffer. Vortex the solution vigorously until the Triton X-100 dissolves completely. Then, add 10 ul of the Protease Inhibitor Cocktail, a 1:100 dilution. Vortex and store the Lysis Buffer containing 1% Triton X-100 on ice. Note: Triton X-100 is very viscous, therefore, cut the pipette tip to enlarge the opening and allow unrestricted flow of the Triton X-100. Cholera Toxin B Subunit–Peroxidase (CTB-HRP) Solution – Reconstitute the lyophilized CTB-HRP by adding 50 ul of ultrapure water to the vial. The reconstituted CTB–HRP Solution can be stored at 4 centigrade for at least 6 months. Diluted CTB–HRP Solution - Before use, prepare the Diluted CTB–HRP Solution by diluting a sample of the Cholera Toxin B Subunit–Peroxidase Solution 1:500 in PBS. Keep on ice. Do not store the Diluted CTB–HRP Solution. For a 10 cm tissue culture plate prepare 5 ml of Diluted CTB–HRP Solution (add 10 ul of the CTB-HRP Solution to 5 ml PBS).
Separation Protocol	Sample Preparation The procedure described is for a single gradient. It requires one or two 10 cm tissue culture plates of cultured cells (80–90% confluence) and varies between cell lines. For example, for A431, HeLa, NIH 3T3, HdFn, HFF, and BHK cell lines, one 10 cm plate is sufficient. For BAEC and HEK 293T cell lines, two 10 cm plates are required, and for the PC-12 cell line, a 150 cm flask is required (approximately three 10 cm plates). For cells in suspension, K562, Jurkat, and U937, 2-5×10^7 cells are needed. All the reagents (PBS, Lysis Buffer containing 1% Triton X-100, OptiPrep, and OptiPrep gradient layers) and the tubes (ultracentrifuge and microcentrifuge tubes)

should be ice cold. CTB-HRP labeled samples cannot be used for immunodetection with peroxidase conjugated antibodies.

A. From Adherent Cells

Perform steps 2-9 in a cold room

1. Pre-cool on ice, 1.5 ml and 2 ml microcentrifuge tubes for lysate and gradient preparation, respectively. Pre-cool the ultracentrifuge.
2. Set the cell plate(s) on ice. If the CTB-HRP binding test is to be performed, continue with step 3, otherwise, go to step 5.
3. Aspirate the medium, wash twice with ice-cold PBS (7 ml each time), and add 5 ml of Diluted CTB-HRP Solution. Gently shake the plate(s) on ice for 1 hour.
4. Aspirate the Diluted CTB–HRP Solution from the plate(s).
5. Wash the plate(s) twice with ice-cold PBS (~7 ml each time).
6. Aspirate the PBS from the plate (if several plates are used for loading on the same gradient, aspirate the PBS from the first plate only) and place the plate on ice at a 90° angle to allow the collection of residual PBS.
7. Add 1 ml of Lysis Buffer containing 1% Triton X-100 to the plate. Scrape the cells using a rubber policeman. If more than one plate is used for separation on a single gradient, repeat step 6 (PBS collection) for each plate and scrape the cells using the cell lysate from the previously scraped plate.
8. Transfer the cell lysate to a pre-cooled, marked microcentrifuge tube.
9. Incubate on ice for at least 30 minutes. Save 50 ul of the lysate as a positive control. Keep it on ice. From this stage the work can be performed on ice in the laboratory.

B. From Cells in Suspension

Perform steps 2-9 in a cold room

1. Pre-cool on ice, 50 ml tubes and 2 ml microcentrifuge tubes for lysate and gradient preparation, respectively. Pre-cool the centrifuge and the ultracentrifuge.
2. Collect the cells in the pre-cooled 50 ml tube(s), and centrifuge the cells at 450 ×g for 5 minutes at 4 centigrade. If the CTB-HRP binding test is to be performed, continue with step 3, otherwise, go to step 6.

3. Aspirate the medium, suspend the cell pellet gently in ice-cold PBS (10 ml), centrifuge at 450× g for 5 minutes at 4 centigrade, and aspirate the PBS. Repeat this step once.
4. Gently suspend the cell pellet in 2 ml of Diluted CTB–HRP Solution. Gently shake the tube(s) horizontally on ice for 1 hour.
5. Centrifuge the tube(s) at 450×g for 5 minutes at 4 centigrade and aspirate the Diluted CTB–HRP Solution from the tube(s).
6. Wash the cells twice as detailed in step 3.
7. Suspend the cell pellet in 1 ml of Lysis Buffer containing 1% Triton X-100.
8. Transfer the cell lysate to a pre-cooled, marked microcentrifuge tube.
9. Incubate on ice, in the cold room, for at least 30 minutes. Save 50 ul of the lysate as a positive control. Keep it on ice. From this stage the work can be performed on ice in the laboratory.

Density Gradient Preparation
The density gradient is made of 5 layers of OptiPrep with different concentrations: 35%, 30%, 25%, 20% and 0%. The lower layer (35% OptiPrep) contains the cell lysate. Work with pre-cooled Lysis Buffer, OptiPrep (60% w/v), OptiPrep gradient layers, and 2 ml microcentrifuge tubes.
1. Prepare the 5 solutions that will form the OptiPrep gradient layers according to Table 1. Mix each one well by vortexing. Keep the prepared solutions on ice.
Note: In order to create an OptiPrep layer, which contains precisely 35% OptiPrep, the volume of cell lysate in tube number 1 should be exactly 0.84 ml.
2. Put 2 ml of gradient layer 1 (35% OptiPrep containing the cell lysate) at the bottom of the pre-cooled ultracentrifuge tube.
3. Place each OptiPrep gradient layer over the other in order using a Pasteur pipette. It is recommended to use an electric pipettor.
4. Balance the ultracentrifuge tubes.
5. Centrifuge at 200,000×g using TFT 65.13 rotor (Kontron Instruments, fixed angle or equivalent) for 4 hours at 4 centigrade.

6. Take the tubes carefully out of the ultracentrifuge and put them on ice.
7. Mark 9 microcentrifuge tubes from 1-9. Tube number 1 will be used for the lowest % of the gradient (the top of the ultracentrifuge tube).

Collection of Fractions

1. Mark a line on a Pasteur pipette to indicate a 1 ml volume. Connect the pipette to an electric pipettor.
2. Carefully collect 1 ml fractions from top to bottom of the ultracentrifuge tube and transfer each fraction to a marked microcentrifuge tube. The number of total collected fractions can vary between 7-9.
3. Keep the fractions on ice for later use. Note: The CTB-HRP and the caveolin-1 are found in fractions 2-5 counting from the top. The samples can be stored at -20 centigrade for up to one month.

Gradient Fraction Analysis

CTB-HRP detection by dot blot analysis

1. Cut two Whatman 3 mm paper rectangles (10×4 cm) and soak in PBS.
2. Cut a nitrocellulose membrane of the same size. Mark ten 2 ×2 cm squares.
3. Place the wet Whatman paper rectangles on a working area and place the nitrocellulose membrane on top of them. The wet Whatman paper keeps the nitrocellulose membrane moist.
4. Load 2-3 ul of each gradient fraction as well as 2-3 ul of the original lysate on the nitrocellulose membrane squares.
5. Wait until the drop absorbs and let the nitrocellulose membrane dry at room temperature.
6. Wash the nitrocellulose membrane once briefly with PBS.
7. Prepare a Chemiluminescent Peroxidase Substrate solution, enough to cover the membrane according to the Chemiluminescent Peroxidase Substrate instructions, and apply on the nitrocellulose membrane.
8. Drain the membrane of excess substrate solution, wrap in plastic wrap, and expose to X-ray film. An initial 10 second exposure will indicate the need for different exposure times.

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Detection of Caveolin-1

Note: CTB-HRP labeled samples cannot be used for immunodetection with peroxidase conjugated antibodies.

A. Dot blot detection of the caveolae isolated fractions

1. Perform steps 1-5 of Gradient Fraction Analysis for dot blot analysis with CTB-HRP detection.
2. Incubate the membrane with a blocking solution. Shake for 1 hour at room temperature.
3. Dilute the Anti-Caveolin-1 1:5,000 in blocking buffer, cover the membrane with the diluted antibody solution, and shake the membrane for one hour at room temperature.
Note: This step can be extended to an overnight incubation in a cold room.
4. Wash the membrane with PBST 3 times for 10 minutes each, at room temperature.
5. Dilute the secondary antibody (anti-Rabbit IgG– Peroxidase Conjugate) 1:5,000 in PBS, cover the membrane with the diluted secondary antibody solution, and incubate the membrane for 1 hour at room temperature.
6. Wash the membrane with PBST 3 times for 10 minutes each, at room temperature.
Perform steps 7-8 of Gradient Fraction Analysis for dot blot analysis with CTB-HRP detection.

B. Western blot detection of caveolin-1 in the caveolae isolated fractions.

1. Run each gradient fraction on SDS-PAGE.
Note: Caveolin-1 is a small protein (21–24 kDa).
2. Transfer to a nitrocellulose membrane according to the instructions of the transfer device.
3. Follow steps 2-6 in the section for Dot blot detection of the caveolae isolated fractions.

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