

Product Information

Caveolae/Rafts Isolation Kit

Product Code **CS0750**

Storage Temperature $-20\text{ }^{\circ}\text{C}$

TECHNICAL BULLETIN

Product Description

Caveolae and lipid rafts are important cholesterol and sphingolipid-rich structures representing subcompartments or microdomains of the plasma membrane. Caveolae and lipid rafts were first functionally implicated in cellular transport processes, and more recently in signal transduction related events.¹⁻⁴

The Caveolae/Rafts Isolation Kit provides an easy method for obtaining fractions enriched with caveolea/rafts proteins from tissue culture cells. The method for the isolation of caveolae and rafts is based on the insolubility of these structures in ice cold TRITON™ X-100^{5,6} followed by their separation on an OptiPrep® density gradient.

The kit contains the required reagents for cell lysis and density gradient isolation of fractions enriched with caveolea/rafts proteins. The kit also includes two reagents for detection of caveolae and rafts markers: cholera toxin B subunit–peroxidase conjugate (CTB-HRP) that binds to the G_{M1} ganglioside receptor and anti-Caveolin-1 antibody that detects caveolin-1.

The kit was tested on A431, HeLa, NIH 3T3, HdFn, HFF, BAEC, PC-12, BHK, HEK 293T, Jurkat, HL-60, NSO, K562, and U937 cell lines.

Components

The kit is sufficient for ~20 density gradient preparations. The CTB-HRP is sufficient for 5 assays (addition to five 10 cm tissue culture plates).

Lysis Buffer 100 ml
Product Code L7667

Cholera Toxin B Subunit from *Vibrio cholerae*–
Peroxidase Conjugate 50 µg
Product Code C3741

Protease Inhibitor Cocktail 1 ml
for use with mammalian cell and tissue extracts
Product Code P8340

Anti-Caveolin-1 50 µl
antibody produced in rabbit
Product Code C3237

TRITON X-100 2 ml
Product Code X100

OptiPrep Density Gradient Medium 100 ml
Product Code D1556

Equipment Required But Not Provided

- Ultracentrifuge
- Rotor TFT 65.13 (Kontron Instruments, fixed angle or equivalent)
- Cell scraper (Product Code C5981)
- Ultracentrifuge tubes suitable to the rotor (volume of ~10 ml)
- Horizontal shaker
- 2 ml microcentrifuge tubes (Product Code T2795 or equivalent)
- Pasteur pipettes, 9 inch (Product Code Z190616)
- Centrifuge tubes, 50 ml (for cells in suspension, Product Code CLS430290)

Reagents and Equipment Required for Analysis of Gradient Fractions by Dot Blot or Western Blot Analysis

- Nitrocellulose membrane for blotting (Product Code N8379 or equivalent)
- Chemiluminescent Peroxidase Substrate-1 (Product Code CPS160 or equivalent)
- Phosphate Buffered Saline with TWEEN® 20 (PBST, Product Code P3563)
- Western Blocker Solution (Product Code W0138)

- Secondary antibody, Anti-Rabbit IgG (whole molecule)–Peroxidase Conjugate (Product Code A9169 is recommended)
- Film - Kodak® BioMax XAR-5 (Product Code F5388)
- Dulbecco's Phosphate Buffered Saline (PBS, Product Code D8537)

Precautions and Disclaimer

This product is for R&D use only, not for drug, household, or other uses. Please consult the Material Safety Data Sheet for information regarding hazards and safe handling practices.

Preparation Instructions

It is recommended to use ultrapure (17 MΩ·cm or equivalent) 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 µl of TRITON X-100 to 1 ml of Lysis Buffer (Product Code L7667). Vortex the solution vigorously until the TRITON X-100 dissolves completely. Then, add 10 µl of the Protease Inhibitor Cocktail (Product Code P8340), 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 µl of ultrapure water to the vial. The reconstituted CTB–HRP Solution can be stored at 4 °C 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 µl of the CTB-HRP Solution to 5 ml PBS).

Storage/Stability

The Caveolae/Rafts Isolation Kit is shipped on dry ice. The Protease Inhibitor Cocktail (Product Code P8340) and the Anti-Caveolin-1 antibody (Product Code C3237) should be stored at –20 °C. It is recommended to store the antibody in working aliquots. After thawing, the aliquot in use can be stored at 2–8 °C for at least 4 weeks. The Lysis Buffer (Product Code L7667) and the CTB-HRP (Product Code C3741) should be stored at 2–8 °C. The TRITON X-100 (Product Code X100) and the OptiPrep Density Gradient Medium (Product Code D1556) should be stored at room temperature.

Procedure

Principle of Isolation

Caveolae/rafts have a unique feature of relative resistance to solubilization in an ice cold TRITON X-100 solution. This feature is used for their isolation. Since the procedure is highly temperature dependent, the work should be performed in the cold room. Note that at 8 °C the Caveolae/Rafts proteins may already be soluble in the TRITON X-100 solution and will not float up in the gradient.

A detergent treated cell lysate is mixed with OptiPrep, placed at the bottom of an ultracentrifuge tube, and layered with OptiPrep layers of decreasing concentration creating a discontinuous gradient. Following centrifugation, the lipid rafts and the caveolae, which have a relatively low density, float away from the TRITON X-100 soluble proteins, and are enriched at the lower concentrations of the OptiPrep gradient. Caveolae microdomains and lipid rafts are recovered as a fine dense band at the border of 20% (w/v) and 30% (w/v) OptiPrep layers after ultracentrifugation at ~200,000 x g for 4 hours. Proteins that are part of the caveolae/rafts or bound to these structures are present in the caveolae/rafts enriched fraction.

Two markers are used for the identification of caveolae and rafts:

1. G_{M1} ganglioside receptor (detected by CTB-HRP binding to this receptor) - The detection of the G_{M1} receptor by CTB-HRP requires binding of CTB-HRP to intact cells and, thus, should be performed before cell lysis.

Note: Not every cell line binds CTB-HRP.

2. Caveolin-1 (detected by the anti-caveolin-1 antibody) - Caveolin-1, a marker protein for the caveolae structure, is a 21-24 kDa integral membrane protein that associates with caveolae related domains by direct interaction with cholesterol and glycosphingolipids.^{7, 8} The caveolin-1 detection is performed after the gradient separation.

Note: There are cells that do not express caveolin-1, such as cell lines derived from white blood cells.^{9,10} In oncogenically transformed cells the caveolin cytosolic pool (the TRITON X-100 soluble fraction) may undergo changes.¹¹

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, $\sim 2\text{-}5 \times 10^7$ 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.

See Appendix for alternative methods of cell lysis.

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 μ l 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 x g for 5 minutes at 4 °C. 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 x g for 5 minutes at 4 °C, 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 x g for 5 minutes at 4 °C 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 μ l 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 (see Table 1). Work with pre-cooled Lysis Buffer, OptiPrep (60% w/v), OptiPrep gradient layers, and 2 ml microcentrifuge tubes.

- 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.

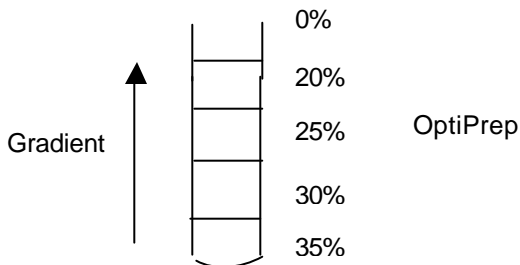
Table 1.
Preparation of OptiPrep Density Gradient Layers

Gradient layer	Final OptiPrep (%)	Cell lysate (ml)	*Lysis buffer (ml)	OptiPrep (ml)	Total volume (ml)
1 (bottom)	35%	0.84	0	1.16	2
2	30%	----	1	1	2
3	25%	-----	1.16	0.84	2
4	20%	-----	1.3	0.7	2
5 (top)	0%	-----	1	0	1

* Can be replaced by Lysis Buffer containing 1% TRITON X-100.

- Put 2 ml of gradient layer 1 (35% OptiPrep containing the cell lysate) at the bottom of the pre-cooled ultracentrifuge tube.
- Place each OptiPrep gradient layer over the other in order (see Figure 1) using a Pasteur pipette. It is recommended to use an electric pipettor.

Figure 1.
Schematic Illustration of the Gradient



- Balance the ultracentrifuge tubes.
- Centrifuge at $\sim 200,000 \times g$ using TFT 65.13 rotor (Kontron Instruments, fixed angle or equivalent) for 4 hours at 4 °C.

- Take the tubes carefully out of the ultracentrifuge and put them on ice.
- 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

- Mark a line on a Pasteur pipette to indicate a 1 ml volume (see Figure 2). Connect the pipette to an electric pipettor.

Figure 2.
Illustration of a Pasteur Pipette with a Mark Indicating a 1 ml Volume.



- 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.
- 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 $-20\text{ }^{\circ}\text{C}$ for up to one month.

Gradient Fraction Analysis

CTB-HRP detection by dot blot analysis

- Cut two Whatman[®] 3 mm paper rectangles (10 x 4 cm) and soak in PBS.
- Cut a nitrocellulose membrane of the same size. Mark ten 2 x 2 cm squares according to Figure 3.

Figure 3.
Schematic Illustration of the Dot Blots for CTB-HRP Binding Analysis.

1	2	3	4	
○	○	○	○	○
○	○	○	○	○

- 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 μ l of each gradient fraction as well as 2-3 μ l 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 (Product Code D8537).
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.

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 (Product Code W0138). Shake for 1 hour at room temperature.
 3. Dilute the Anti-Caveolin-1 (Product Code C3237) 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 (Product Code P3563) 3 times for 10 minutes each, at room temperature.
 5. Dilute the secondary antibody (anti-Rabbit IgG-Peroxidase Conjugate, Product Code A9169) 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 (Product Code P3563) 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.

Appendix

Alternative methods for cell lysis:

Sodium Bicarbonate as Lysis Buffer

This method will solubilize and detect proteins, which are peripherally associated with the membrane.^{7,11}

Reagents

- Sodium bicarbonate (Na_2CO_3 , Product Code S8875)
- Dounce tissue grinder 2 ml (Product Code D8938 for set, Product Code P0485 for Dounce tissue grinder replacement)
- Sonicator

Procedure

Perform the cells lysis in the cold.

1. Prepare a 0.5 M sodium bicarbonate solution in water at pH 11 (this is the pH of the solution as prepared).
2. Perform steps 1-9 as described in the Sample Preparation section, using 0.5 M sodium bicarbonate as the lysis buffer.
3. For a further homogenization use a loose fitting 3 ml Dounce homogenizer for ~10 strokes. Transfer the homogenate to 1.5 ml microcentrifuge tube.
4. Sonicate the lysate for 10 seconds at the maximal energy.
5. Continue the procedure as described in Density Gradient Preparation section.
Note: The gradient layers should be prepared according to Table 1 using 0.25 M sodium bicarbonate solution instead of Lysis Buffer.

Saponin (control experiment)

The addition of saponin to the lysate disrupts lipid rafts and prevents the flotation of the rafts proteins in the density gradient, thus affecting the fractionation pattern. The fractions containing these proteins will be found at the bottom of the ultracentrifuge tube.^{12,13}

Reagent

- Saponin (Product Code S4521)

Procedure

1. Prepare a stock solution of 10% (w/v) Saponin in Lysis Buffer.
2. Perform steps 1-6 as described in the Sample Preparation section.
3. Add 0.9 ml of Lysis Buffer containing 1% TRITON X-100 to the plate/cell pellet. Scrape the cells using a rubber policeman (for adherent cells) or suspend the cell pellet (for cells in suspension), as described in step 7 in the Sample Preparation section.
4. Transfer the lysate to a pre-cooled, marked microcentrifuge tube.
5. Add 0.1 ml of the pre-cooled 10% (w/v) Saponin in Lysis Buffer to give a final concentration of 1% saponin.
6. Incubate on ice in a cold room for at least 30 minutes.
7. Continue with the procedure in the Density Gradient Preparation section.

Note: The gradient layers should be prepared according to Table 1 using a lysis buffer containing 1% saponin.

References

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Troubleshooting Guide

Problem	Possible cause	Solution
No detection (or a very weak signal) of caveolin-1 in the floated fractions	The amount of caveolin-1 in the sample is too low.	Use more than one 10 cm plate for separation on a single gradient.
		Decrease the dilution of the Anti-Caveolin-1 antibody (Product Code C3237).
		Using the dot blot detection, load up to 4 μ l of the floated fractions on the nitrocellulose membrane.
A high background	The first antibody (Anti-Caveolin-1) is too concentrated.	Perform a higher dilution of the Anti-Caveolin-1 antibody (Product Code C3237). Dilute up to 1:10,000.
	The extract is too concentrated.	Using the dot blot detection, load 1 μ l of the floated fractions on the nitrocellulose membrane.
		Use a lower amount of extract for separation on a single gradient.
No detection of CTB-HRP	The cell line chosen does not contain G _{M1} receptor capable of binding CTB-HRP.	In this specific cell line, the CTB-HRP cannot be used as a marker for caveolae/rafts.

EB,MAM 02/06-1

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