A Simplified Method for the Preparation of Detergent-free Lipid Rafts

Jennifer L. Macdonald and Linda J. Pike*
Washington University School of Medicine
Department of Biochemistry and Molecular Biophysics
660 So. Euclid Ave, Box 8231
St. Louis, MO 63110

*Person to whom correspondence should be addressed
Telephone: 314 362-9502
FAX: 314 362-7183
e-mail: pike@wustl.edu

Running title: Preparation of detergent-free lipid rafts
Abstract

Lipid rafts are small plasma membrane domains that contain high levels of cholesterol and sphingolipids. Traditional methods for the biochemical isolation of lipid rafts involve the extraction of cells with non-ionic detergents followed by the separation of a low density, detergent-resistant membrane fraction on density gradients. Because of concerns regarding the possible introduction of artifacts through the use of detergents, it is important to develop procedures for the isolation of lipid rafts that do not involve detergent extraction. We report here a simplified method for the purification of detergent-free lipid rafts that requires only one short density gradient centrifugation, but yields a membrane fraction that is highly enriched in cholesterol and protein markers of lipid rafts with no contamination from non-raft plasma membrane or intracellular membranes.

Key words: caveolin, cholesterol, EGF receptors, flotillin, Gq
Introduction

Lipid rafts are low density plasma membrane domains that are involved in a number of cellular processes such as trafficking (1) and cell signaling (2-5). A variety of proteins have been shown to be selectively enriched in lipid rafts. This includes GPI-anchored proteins (6-10) and dually acylated proteins (11-15) that appear to be targeted to rafts as a result of their post-translational modification with lipids. Several transmembrane proteins including flotillin (16), receptor tyrosine kinases (17-19) and G protein-coupled receptors (20-25) have also been shown to be enriched in lipid rafts, though the targeting mechanisms for such proteins are not well-defined. Caveolin, the structural protein of the subclass of lipid rafts known as caveolae (26, 27), is also typically found in low density plasma membrane fractions. Together, these proteins are used as markers for lipid rafts during biochemical fractionation procedures designed to isolate these plasma membrane domains.

Lipid rafts contain high levels of sphingolipids and cholesterol and probably exist in a liquid ordered phase (28). Because of the presence of cholesterol and sphingomyelin as well as the preponderance of saturated acyl chains in lipid rafts (29, 30), the acyl chains in these domains tend to be well-ordered and tightly packed. This physical property gives rise to the known ability of lipid rafts to withstand disruption by non-ionic detergents (6, 31). The high lipid:protein ratio of such detergent-resistant lipid rafts makes them significantly lower in density than other solubilized membrane proteins and allows them to be isolated from other membrane proteins by centrifugation through density gradients.

Early preparations of lipid rafts used 1% Triton X-100 to extract whole cells and the low density, detergent-resistant material was separated from other solubilized membrane fractions by centrifugation on a 5 to 30% sucrose density gradient (6). Subsequently, lipid rafts have been prepared using a variety of other detergents including Lubrol WX, Lubrol PX, Brij 58, Brij 96, Brij 98, Nonidet P40, CHAPS and octylglucoside (32-36). While preparations of detergent-resistant membranes are readily isolated, several observations have raised concerns that extraction of cells with detergent may be generating clusters of raft lipids and proteins that did not exist in the intact cell. For
example, examination of cells grown on coverslips and then extracted with Triton X-100 reveals a continuous membrane sheet pock-marked by large holes (37). Since rafts are thought to be less than 50 nm in diameter (38-40), the residual “detergent-resistant” membrane probably formed from individual rafts that coalesced as a result of the detergent treatment.

To avoid the complications associated with preparing rafts using detergent extraction procedures, several methods have been established for isolating rafts from cells fractionated in the absence of detergent. Song et al (41) sonicated cells in a pH 11 sodium carbonate buffer and isolated caveolae by centrifugation of the lysate in a discontinuous 5%/35%/45% sucrose gradient. While this procedure is relatively easy, the spin time of 16 to 20 hours is long and the resulting raft fraction is significantly contaminated with other membranes. A more careful fractionation of lipid rafts and caveolae was devised by Smart et al (42) who lysed cells in an isotonic buffer containing EDTA and purified plasma membranes on a Percoll gradient. The plasma membranes were then sonicated and the lipid rafts isolated by flotation through a 10% to 20% gradient of OptiPrep. This method results in the production of a relatively clean raft preparation. However, it is time-consuming and yields are poor. Furthermore, there is significant variability from preparation to preparation and from cell type to cell type.

Because of the drawbacks associated with existing procedures for isolating detergent-free lipid rafts, we sought to develop an improved method that would allow the rapid isolation of purified rafts in good yield. We report here that lysis of cells by shearing in an isotonic buffer containing calcium and magnesium permits isolation of a highly purified raft fraction in a single step by flotation in a 0 to 20% OptiPrep gradient.
Experimental Procedures

Materials

OptiPrep was obtained from Granier BioOne. Percoll was obtained from Sigma Chemical Co. CII Total Cholesterol Assay Kit was from Wako. Protease inhibitor cocktail set III was from Calbiochem. Calpain inhibitor I was from Sigma. The polyclonal anti-EGF receptor antibody and polyclonal anti-Gq antibody were from Santa Cruz. The monoclonal anti-transferrin receptor antibody was obtained from Zymed. The monoclonal antibodies against flotillin-1 and annexin II and the polyclonal antibody against caveolin-1 were purchased from Transduction Laboratories. The polyclonal anti-ß-COP antibody was from Sigma and the polyclonal anti-calnexin antibody was from Stressgen. The monoclonal anti-prohibitin antibody was from Neomarkers. The polyclonal antibody against 5’-nucleotidase was obtained from Abgent. The alpha-nucleoporin antibody was the generous gift of Dr. Susan Wente (Vanderbilt University). Horse radish peroxidase-conjugated anti-rabbit IgG was from Pierce. Horse radish peroxidase-conjugated anti-mouse IgG and chemiluminescence reagents were from Amersham.

Cells and tissue culture

Chinese hamster ovary cells (CHO cells) transfected with human EGF receptors and HeLa cells were grown in Ham’s F12 medium containing 10% fetal calf serum. Human breast adenocarcinoma MDA-231 cells were maintained in RPMI1640 containing 10% fetal calf serum. All cells were grown in a humidified incubator in 5% CO2.

Simplified method for the preparation of detergent-free lipid rafts

All procedures were carried out on ice. Four D150 plates of cells were washed and scraped into base buffer (20 mM Tris-HCl, pH 7.8, 250 mM sucrose) to which had been added 1 mM CaCl2 and 1 mM MgCl2. Cells were pelleted by centrifugation for 2 min at 250 x g and resuspended in 1 ml of base buffer containing 1 mM CaCl2, 1 mM MgCl2, and protease inhibitors at final concentrations of: 0.2 mM AEBSF, 1 µg/ml aprotinin, 10 µM bestatin, 3 µM E-64, 10 µg/ml leupeptin, 2 µM pepstatin, 50 µg/ml calpain inhibitor
I. The cells were then lysed by passage through a 22 g x 3” needle 20 times. Lysates were centrifuged at 1000 x g for 10 min. The resulting post-nuclear supernatant was collected and transferred to a separate tube. The pellet was again lysed by addition of one ml base buffer plus divalent cations and protease inhibitors followed by sheering 20 times through a needle and syringe. After centrifugation at 1000 x g for 10 min, the second post-nuclear supernatant was combined with the first.

An equal volume (2 ml) of base buffer containing 50% OptiPrep was added to the combined post-nuclear supernatants and placed in the bottom of a 12 ml centrifuge tube. An 8 ml gradient of 0 to 20% OptiPrep in base buffer was poured on top of the lysate which was now 25% in OptiPrep. Gradients were centrifuged for 90 min at 52,000 x g using an SW-41 rotor in a Beckman ultracentrifuge. After centrifugation, cloudiness could be seen throughout the gradient. A diffuse band was observed about one-third of the way down the gradient and a distinct band was apparent at the interface of the 20% end of the gradient and the 25% OptiPrep bottom layer. Gradients were fractionated into 0.67 ml fractions and the distribution of various proteins was assessed by Western blotting. Total protein in each fraction was determined by precipitation Lowry (43). Total cholesterol was determined using the Wako CII Total Cholesterol assay kit.

Preparation of other types of detergent-free rafts

Detergent-free rafts were prepared using the carbonate step gradient method of Song et al (41). Briefly, two D150 plates of cells were washed and scraped into phosphate-buffered saline. After pelleting the cells for 2 min at 250 x g, 2 ml 500 mM sodium carbonate, pH 11.0 containing protease inhibitors as above were added to the cell pellet. Cells were lysed by 20 strokes in a Dounce homogenizer using a tight fitting pestle followed by 10 passages through a 23g needle and finally sonication 3 times for 15 seconds in a Branson Sonifier 250. The homogenate was mixed with an equal volume (2 ml) of MES-buffered saline (25 mM MES, pH 6.5, 150 mM NaCl) containing 90% sucrose and placed in the bottom of a test tube. Four ml of MES-buffered saline
containing 35% sucrose was layered on top followed by 4 ml of MES-buffered saline containing 5% sucrose. Gradients were centrifuged for 16 hr at 175,000 x g in an SW41 rotor. Tubes were fractionated into twelve 1 ml fractions.

Rafts were also prepared by the sequential linear gradient method of Smart et al (42). Four D150 plates of cells were washed and scraped into buffer A (20 mM Tris, pH 7.8, 0.25 M sucrose, 1 mM EDTA) containing protease inhibitors as above. Cells were lysed in 1 ml buffer A using 10 strokes in a Dounce homogenizer followed by 10 passages through a 23 g needle. The lysate was centrifuged for 10 min at 1000 x g and the supernatant collected. The pellet was lysed as before in 1 ml buffer A. After centrifugation, the two post-nuclear supernatants were combined and placed on top of 8 ml 30% Percoll and centrifuged for 30 min at 84,000 x g in a Beckman Ti75 rotor. A visible band approximately one-third of the distance down the tube was collected and used as the plasma membrane fraction. This fraction was adjusted to 2 ml with buffer A and then sonicated with 6 pulses at 50% duty cycle using a Branson Sonifier 250. After a one minute rest, the sample was sonicated with a second set of 6 pulses. The procedure was repeated for a total of 18 one-second pulses. The sonicate was mixed with 1.8 ml buffer A containing 50% OptiPrep plus 0.2 ml buffer A. An 8-ml gradient of 10% to 20% Opti-Prep in buffer A was poured on top and the tubes centrifuged for 90 min at 52,000 x g. Gradients were fractionated and protein distribution analyzed by Western blotting.

Results

Figure 1 compares the distribution of several membrane proteins in two earlier detergent-free lipid raft membrane preparations. The distribution of marker proteins in the carbonate step gradient of Song et al. (41) is shown on the left while the distribution of the same proteins in the sequential linear gradients procedure of Smart et al (42) is shown on the right. As can be seen from the figure, in the carbonate step gradient procedure, raft proteins including flotillin, caveolin, Gq and the EGF receptor are present.
in fractions 4 and 5, which represent the interface between the 5% and 35% sucrose pads. However, this “raft” fraction also contains a significant amount of transferrin receptor, a non-raft plasma membrane protein, and β-COP, a Golgi marker. The fraction appears to be relatively free of contamination by ER membranes as evidenced by the absence of calnexin from fractions 4 and 5.

In contrast to the carbonate step gradient rafts, the low density membranes derived from the sequential linear gradient procedure are not contaminated with either ER or Golgi markers. A significant portion of these intracellular membranes are removed during the initial plasma membrane purification in the Percoll gradient (data not shown) and the remainder are readily separated from the raft membranes in the second OptiPrep gradient. The raft protein markers, flotillin, caveolin, and Gq were broadly distributed in the gradient, but ~ one-third of each was found in the upper third of the gradient. Approximately one-quarter of the EGF receptor was also found in the lowest density fractions. If only the lightest 5 fractions of the OptiPrep gradient are pooled, a relatively clean raft fraction can be obtained. However, the preparation requires a full day to complete and yields are often poor. More importantly, there is significant day-to-day and cell-to-cell variability in the fraction of raft markers that can be recovered in the low density portion of the gradient.

Since the method of Smart et al (42) provided a cleaner preparation of rafts, we used this as the starting point for our method and modified the procedure to optimize for purity, yield and ease of preparation. The method that was developed omits the initial purification of plasma membranes over the Percoll gradient and employs a single OptiPrep gradient to separate lipid rafts from other membrane fragments. The key to obtaining these separations was the removal of EDTA and the inclusion of calcium and magnesium in the washing and lysis buffer. However, divalent cations were absent from the OptiPrep gradient used to separate the membrane fractions. Figure 2 shows the
distribution of several membrane proteins across the density gradient in this modified procedure.

Most raft membrane markers were found primarily in fractions 1 through 3 of the gradient. This included ras, Gq, GPI-anchored 5’-nucleotidase, and the EGF receptor. Flotillin was recovered over a somewhat broader density range, but a significant fraction of this marker was still found in the lightest fractions of the gradient. Flotillin has been reported to be present in cellular compartments other than lipid rafts (44-46). Thus, its presence throughout the gradient probably reflects its varied intracellular distribution. These raft fractions correspond to a concentration of 0 to 5% OptiPrep. Because the base buffer contains ~9% sucrose, the material recovered in these fractions is similar in density to the membranes isolated using detergent extraction and sucrose density gradient centrifugation (6).

The peripheral membrane protein, annexin II, was recovered in both the low density raft fractions and in the higher density portion of the gradient. Interestingly, although some caveolin was found in the top three fractions, this marker protein was recovered primarily in fractions 7 through 10, well outside the main lipid raft fractions. Importantly, the transferrin receptor, a marker for non-raft plasma membrane was well-separated from the major lipid raft fractions, indicating that non-raft plasma membrane did not significantly contaminate the major lipid raft fractions. Furthermore, β-COP and calnexin, the Golgi and ER markers, respectively, were also well-separated from the lipid rafts as were prohibitin, a marker for mitochondria, and alpha-nucleoporin, a marker for nuclear membranes. Thus, this procedure cleanly separates intracellular membranes from plasma membrane lipid rafts.

To determine the reproducibility of the fractionation method, rafts were prepared on three separate days using the above protocol. Fractions were analyzed for marker proteins by Western blotting and the blots quantitated to determine the fraction of the various markers that were recovered in the lipid raft fraction (fractions 1 through 3). The
results are shown in Figure 3. On average, nearly 70% of the EGF receptor and 60% of Gq was recovered in the lipid raft fractions. A somewhat lower fraction, 40% to 50%, of ras and flotillin was found in the raft fractions while about 35% of 5’-nucleotidase and annexin II was recovered in rafts. All non-raft markers including those for plasma membrane and intracellular organelles were present at less than 5%. Daily variation in recovery was generally 15% or less. Thus, the method reproducibly recovers raft proteins and purifies them away from non-raft markers.

Figure 4 shows the distribution of total protein and cholesterol across the OptiPrep gradient. As can be seen from the figure, relatively little protein was present in the raft fractions. The bulk of the protein was found at the bottom of the gradient. Cholesterol levels in the low density fractions were high. When plotted as µg cholesterol/mg protein, it can be seen that the light fractions are significantly enriched in this sterol as compared to the higher density fractions. The protein yield in the raft fractions was approximately twice that obtained using the method of Smart et al (42). Since the amount of cholesterol per mg protein is similar in the two preparations (~900-1000 nmol/mg protein), the level of purity of the two preparations is similar. Thus, we estimate that the recovery of raft material in our preparations is about twice that obtained using the Smart et al. method.

The one-step OptiPrep gradient separation method was applied to two additional cell types, MDA-231 and HeLa cells, to determine whether the procedure produced equivalently good separation and recovery of lipid rafts in other cell systems (Figure 5). In both cell lines, the lipid raft marker proteins floated to fractions 2 through 5, somewhat denser than those found in CHO cells. However, the rafts were still well-separated from non-raft plasma membranes marked by the transferrin receptor. In addition, markers for intracellular membranes were also distributed well away from the raft fractions. Thus, the procedure yielded similar results in terms of separation of rafts from non-raft membranes in a other cell types.
Discussion

We report here on a new method for the isolation of detergent-free lipid rafts from cultured cell lines. The procedure represents a simplification of the methodology of Smart et al (42). While this earlier method generates a fairly clean raft fraction, it requires three ultracentrifugation steps and a full 8 hr to produce a concentrated raft fraction. In addition, we have observed that the sonication step prior to the OptiPrep gradient introduces significant variability into the preparation. Over-sonication results in the recovery of all proteins in the high density portion of the gradient whereas under-sonication fails to separate the raft membranes from the plasma membrane. The amount of sonication necessary to achieve good separation varies from cell line to cell line and also differs with different growth or treatment conditions. This lack of consistency is the major impediment in applying this methodology to the study of lipid rafts.

Our method requires just one short (90 min) ultracentrifugation and omits the sonication step. Thus, it is much faster than the earlier methods. In addition, because there is only one fractionation step, losses are reduced and yields are significantly increased. Analysis of protein marker distribution can easily be accomplished using half as many cells (two D150 plates) as are required for the method of Smart et al (42). If a concentrated raft fraction is desired, this is readily achieved by separating the lysates on a 5% to 20% OptiPrep gradient rather than the 0 to 20% OptiPrep gradient used here. The narrower gradient results in the recovery of all raft markers in fraction 1 and does not affect the separation from other cellular membranes.

Most importantly, this new methodology yields consistent results. Raft markers are always separated from non-raft plasma membrane and intracellular membrane markers and the fractional recovery of a raft marker in the low density fractions is reproducible from experiment to experiment. Membranes from different cell lines appear to have different average densities, thus the rafts and other membranes are recovered at slightly higher or slightly lower positions in the density gradients. However, their relative
separation is similar and the recovery of raft markers in the raft fractions is consistently high. At most a minor adjustment in the boundaries of the OptiPrep gradient would be necessary to produce an optimal separation of rafts from other membranes in different cell lines.

The key change in the protocol was the removal of EDTA from the washing and lysis buffers and the addition of calcium and magnesium. Lysing the cells in buffer containing EDTA followed by separation on a 0 to 20% OptiPrep gradient did not lead to the separation of rafts from the plasma membrane. The reason for the difference in the behavior of lipid rafts under these lysis conditions is not clear, but it is possible that rafts are stabilized by divalent cations, making their isolation easier.

The rafts isolated using the new method exhibit the characteristics typical of this membrane domain. The purified rafts are highly enriched in cholesterol, a key lipid component of such domains. They are also enriched in traditional raft marker proteins including the GPI-anchored protein, 5’-nucleotidase, and the acylated protein, Goq. The rafts also contain transmembrane domain proteins known to be present in lipid rafts, such as flotillin (16) and the EGF receptor (18). Annexin II, a peripheral membrane protein previously shown to be raft-associated, (47) was also found in the raft fractions. The non-raft plasma membrane marker, transferrin receptor, as well as markers for intracellular membranes (β-COP, calnexin, prohibitin, nucleoporin) were excluded from the raft fraction. Thus, this procedure appears to yield a clean fraction of membranes that has all the characteristics of lipid rafts.

Interestingly, caveolin was recovered in fractions of a significantly higher density than other raft marker proteins. The basis for this separation is not clear, but it is possible that the association of caveolae with the actin cytoskeleton is not disrupted by the lysis procedure resulting in the generation of relatively heavy caveolar membranes. It is noteworthy, that this is the first biochemical isolation of lipid rafts in which they were physically separated from caveolae without recourse to immunoaffinity purification.
This may therefore be a useful procedure for determining whether a protein partitions into lipid rafts or caveolae.

In summary, we have developed a simple and rapid method for the isolation of lipid rafts without employing detergents. The availability of a procedure for isolating large amounts of detergent-free lipid rafts will permit easier and more extensive biochemical characterization of these domains.

**Acknowledgments**

This work was supported by National Institutes of Health grant number GM64491 to LJP.
References


of Caveolin Does Not Alter the Lipid Composition of these Domains. *Biochem. 41*, 2075-2088.


Footnotes

Abbreviations used are: EGF, epidermal growth factor, ER, endoplasmic reticulum,
**Figure Legends**

**Figure 1.** *Distribution of membrane proteins in previous detergent-free protocols.* Raft preparations were isolated from CHO cells as described in Materials and Methods. A 100 µl aliquot of each gradient fraction was analyzed by SDS polyacrylamide gel electrophoresis followed by Western blotting for the indicated protein. Panel A, protein distribution in the carbonate step gradient procedure of Song et al. (41). Panel B, protein distribution in the Percoll/OptiPrep linear gradient procedure of Smart et al (42). Flot, flotillin; Cav, caveolin, EGFR. EGF receptor, TfR, transferrin receptor; Calnex, calnexin.

**Figure 2.** *Distribution of membrane proteins in new detergent-free protocol.* CHO cells were lysed and the membranes separated as described in Materials and Methods. A 100 µl aliquot of each gradient fraction was analyzed by SDS polyacrylamide gel electrophoresis followed by Western blotting for the indicated protein. Annex, annexin II; 5’-NT, ecto-5’-nucleotidase. All other abbreviations are as in Figure 1.

**Figure 3.** *Percent recovery of membrane proteins in the raft fraction.* Lipid rafts from CHO cells were prepared using the simplified method and fractions analyzed for marker proteins as in Figure 2. The Western blots were scanned and quantitated using ImageJ. The percent recovery of a protein in the raft fraction was calculated by adding the amount of a marker protein in fractions 1 through 3 and dividing this by the total amount of that protein recovered in all 18 fractions. Values represent the mean ± S.D. from three separate experiments.

**Figure 4.** *Distribution of protein and cholesterol across the OptiPrep gradient.* Protein and cholesterol content of each fraction were determined as indicated in Materials and Methods. Closed circles, total protein content of each fraction; open circles, µg cholesterol/mg protein in each fraction.

**Figure 5.** *Application of the detergent-free raft isolation procedure to MDA-231 and HeLa cells.* MDA-231 and HeLa cells were lysed and membranes separated as outlined
in Materials and Methods. A 100 µl aliquot of each gradient fraction was analyzed by SDS polyacrylamide gel electrophoresis followed by Western blotting for the indicated protein.
Figure 1

A. Carbonate step

B. Two sequential gradients

<table>
<thead>
<tr>
<th>Protein</th>
<th>A1</th>
<th>A2</th>
<th>A3</th>
<th>A4</th>
<th>A5</th>
<th>A6</th>
<th>A7</th>
<th>A8</th>
<th>A9</th>
<th>A10</th>
<th>A11</th>
<th>A12</th>
</tr>
</thead>
<tbody>
<tr>
<td>Flot</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cav</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Gq</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>EGFR</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Tfr</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Calnex</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>βCOP</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Protein</th>
<th>B1</th>
<th>B2</th>
<th>B3</th>
<th>B4</th>
<th>B5</th>
<th>B6</th>
<th>B7</th>
<th>B8</th>
<th>B9</th>
<th>B10</th>
<th>B11</th>
<th>B12</th>
<th>B13</th>
</tr>
</thead>
<tbody>
<tr>
<td>Flot</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cav</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Gq</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>EGFR</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Tfr</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Calnex</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>βCOP</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
Figure 2

Ras
Gq
5'-NT
Flot
EGFR
Cav
Annex
Tfr
β-COP
Calnex
Prohibitin
Nucleoporin

1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18
Figure 4

The graph shows the relationship between Total Protein (µg) and Fraction No. along with the concentration of cholesterol per mg protein. The data points are marked with different symbols and lines to distinguish between Total Protein and cholesterol.
Figure 5

<table>
<thead>
<tr>
<th>Protein</th>
<th>MDA-MB-231</th>
<th>HeLa</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ras</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Gq</td>
<td></td>
<td></td>
</tr>
<tr>
<td>5'-NT</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Flot</td>
<td></td>
<td></td>
</tr>
<tr>
<td>EGFR</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cav</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Annex</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Tfr</td>
<td></td>
<td></td>
</tr>
<tr>
<td>β-COP</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Calnex</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Prohibitin</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Nucleoporin</td>
<td>1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18</td>
<td></td>
</tr>
</tbody>
</table>