

***In vitro* Lipid Transfer Assay**

Yan Zhang, Xiaochen Wang*

National Institute of Biological Sciences, Zhongguancun Life Science Park, Beijing, China

*For correspondence: wangxiaochen@nibs.ac.cn

[Abstract] This is a protocol to detect lipid transfer activity of NRF-5, a member of the LPS binding/lipid transfer protein family. The lipid transfer activity is examined by using isotope-labeled cholesterol and liposomes, and tested in two directions (Figure 1): from proteins to liposomes and from liposomes to proteins.

Materials and Reagents

1. PC (Avanti-Polar Lipids)
2. PE (Avanti-Polar Lipids)
3. Cholesterol (Avanti-Polar Lipids, catalog number: 700000)
4. 1,2-Dioleoyl-sn-Glycero-3-Phosphoethanolamine (DOPE)
(Avanti, catalog number: 850725)
5. 1,2-di-(9Z-octadecenoyl)-sn-glycero-3-phosphocholine (DOPC)
(Avanti, catalog number: 770375)
6. 1 mCi (37 MBq) (PerkinElmer, catalog number: NET139001MC)
7. Protein of interest tagged with Flag
8. Tris-HCl (pH 7.4)
9. NaCl
10. Anti-Flag M2 agarose beads (Sigma-Aldrich, catalog number: A2220)
11. Flag peptide (Sigma-Aldrich, catalog number: F3290)
12. Chloroform
13. Wash buffer (see Recipes)
14. Elution buffer (see Recipes)

Equipment

1. Avanti Mini-Extruder (Avanti, catalog number: 610023)
2. Vortexer
3. Centrifuges
4. Rotator

5. Branson tip-sonicator (Cole-parmer Cp750)
6. Scintillation counter (Wallac MicroBeta TriLux, catalog number: 1450-023)

Procedure

A. Preparation of liposomes (room temperature)

1. Liposome in the absence of cholesterol is made by Avanti Mini-Extruder at room temperature. The dried 1.25 mg mix of PC (75%) and PE (25%) is hydrated in 1 ml buffer (50 mM Tris-Cl, 150 mM NaCl). 100 nm unilamellar vesicles are obtained by extrusion as described:

(http://www.avantilipids.com/index.php?option=com_content&view=article&id=185&Itemid=193)

2. Liposomes containing [³H]cholesterol are generated by using a standard sonication procedure.
 - a. Dissolve dried 2.5 mg mix of PC (75%) and PE (25%) in 200 µl chloroform by vortex.
 - b. Take 8 µl of above chloroform dissolved lipid mix and add 0.001 mg [³H]cholesterol (~2% molar mass).
 - c. The chloroform is evaporated and dried under a stream of nitrogen. Longer drying time (4-12 h) can be used to remove any trace of organic solvent.
 - d. The dry lipid film is hydrated by adding 0.5 ml of buffer (50 mM Tris-Cl, 150 mM NaCl). After vortex at room temperature for 20 min, the large multilamellar vesicle suspension is disrupted with a Branson tip-sonicator until the suspension clear. For sonication, the samples are placed on ice and sonicated for 10 min with cycles including 9 sec sonication, 9 seconds interval and 35% input.
 - e. Metal particles from the sonicator tip and undisrupted lipid aggregates are removed by centrifugation at 100,000 x g for 30 min at 4 °C. The resulting hazy supernatant, composed primarily of small unilamellar vesicles, is stored at 4 °C. The liposomes can be stored at this condition for one week.

B. Examine [³H]cholesterol transfer from liposomes to proteins (Figure 1)

Reactions are performed on ice.

1. In the [³H]cholesterol/liposome to protein transfer assay, each reaction contains, in a final volume of 200 µl buffer (50 mM Tris-Cl, 150 mM NaCl, pH 7.4), 4 µg of PC: PE: [³H]cholesterol liposomes, and different amounts of the acceptor protein EGFP-FLAG and EGFP::NRF-5-FLAG (15 and 30 µg) purified from 293T cells (Zhang *et al.*, 2012). EGFP-FLAG is used as the negative control.

2. After incubation for 30 min at 4 °C, each mixture is diluted with 600 µl 50 mM Tris-Cl, 150 mM NaCl, followed by adding 70 µl 50% Flag beads.
3. After incubation for about 1 h at 4 °C on shaker, the Flag beads are washed 4-5 times in 1,000 µl wash buffer on shaker, and bounded [³H]cholesterol is quantified by scintillation counting.

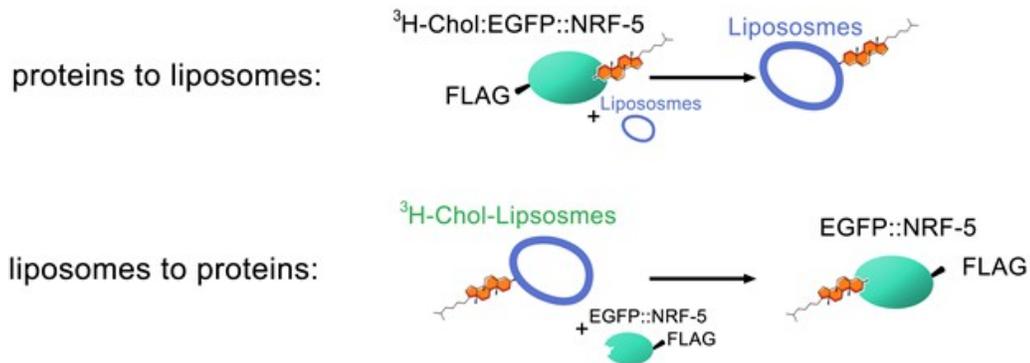


Figure 1. Schematic diagrams of the lipid transfer assay in two directions

- C. Examine [³H]cholesterol transfer from proteins to liposomes (Figure 1)
1. Protein-[³H]cholesterol complex is obtained by incubating EGFP-FLAG or EGFP::NRF-5-FLAG (400 pmol) with [³H]cholesterol (100 pmol) in a final volume of 300 µl buffer (50 mM Tris-Cl, 150 mM NaCl, pH 7.4) for 3 h at 4 °C. The protein-[³H]cholesterol complex is pulled down by incubating with 100 µl 50% Flag beads for 2 h at 4 °C, washing 6 times as above and eluted with 100 µl Flag peptide (100 mg/ml) for two times.
 2. In the protein-to-liposome transfer assay, each reaction contains, in a final volume of 200 µl buffer (50 mM Tris-Cl, 150 mM NaCl, pH 7.4), [³H]cholesterol complexed to either EGFP or EGFP-NRF-5 (40 µl), and different amounts of acceptor PC liposomes (50 and 100 ng).
 3. After incubation for 30 min at 4 °C, each mixture is diluted with 600 µl of buffer (50 mM Tris-Cl, 150 mM NaCl, pH 7.4).
 4. Liposomes are separated by centrifuging at 10,000 x g (hard to detect weight at this stage) for 30 min at 4 °C. The liposomes can be seen as a small white patch at the bottom of the tube.
 5. Wash the liposomes 4-5 times in the buffer (50 mM Tris-HCl, 150 mM NaCl), with 500 µl buffer used in each tube at each time. The liposomes are collected by centrifuge (10,000 x g) after each wash. The amount of [³H]cholesterol transferred to liposomes is determined by scintillation counting.

Recipes

1. Wash buffer
50 mM Tris-Cl, 150 mM NaCl (pH 7.4)
2. Elution buffer
50 mM Tris-Cl, 150 mM NaCl (pH 7.4), Flag peptide

Acknowledgments

This protocol is adapted from Zhang *et al.* (2012) and Infante *et al.* (2008).

References

1. Infante, R. E., Wang, M. L., Radhakrishnan, A., Kwon, H. J., Brown, M. S. and Goldstein, J. L. (2008). [NPC2 facilitates bidirectional transfer of cholesterol between NPC1 and lipid bilayers, a step in cholesterol egress from lysosomes](#). *Proc Natl Acad Sci U S A* 105(40): 15287-15292.
2. Zhang, Y., Wang, H., Kage-Nakadai, E., Mitani, S. and Wang, X. (2012). [C. elegans secreted lipid-binding protein NRF-5 mediates PS appearance on phagocytes for cell corpse engulfment](#). *Curr Biol* 22(14): 1276-1284.