

Isolation of Inner Membrane Vesicles from *E. coli* by Using an Affinity Tag

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[Abstract] This protocol was developed in a project aimed to identify the inner membrane proteins localizing to cell poles in *Escherichia coli* (*E. coli*). By using a known polar protein Tar as a tag, we isolated pole-derived inner membrane vesicles by affinity capture. The specificity of the polar vesicle isolation was confirmed by mass spectrometry that identified more than one hundred proteins, most of which are known inner membrane proteins, including other known polar proteins. This protocol, or if adapted properly by choosing other affinity targets, is well suited to isolate other membrane domains of interest for identification of proteins or lipid composition.

Materials and Reagents

1. Isopropyl β -D-1-thiogalactopyranoside (IPTG)
2. Medium copy plasmid pLP8 for recombinant protein expression (Plac lacIq KanR, Li and Young, 2012)
3. Bacto Tryptone (BD Biosciences, catalog number: 211705)
4. Halt protease inhibitor (Thermo Fisher Scientific, catalog number: 78425)
5. RNase A (Sigma-Aldrich, catalog number: R5503)
6. DNase I (Thermo Fisher Scientific, catalog number: NC9709009)
7. anti-FLAG M2 affinity gel (Sigma-Aldrich, catalog number: A2220)
8. Poly-Prep chromatography column (Bio-Rad Laboratories, catalog number: 731-1550)
9. Micro BCA protein assay kit (Thermo Fisher Scientific, catalog number: 23235)
10. Triton X-100
11. Tween 20
12. Tryptone medium
13. Phosphate buffered saline (PBS) (see Recipes)
14. Phosphate buffered saline with Tween 20 (PBST) (see Recipes)

Equipment

1. Centrifuges for 1.5 ml, 10 ml and 100 ml volumes
2. High shear fluid processor (Microfluidics, model: LV1) or French Press

Procedure

1. Construct an *E. coli* strain expressing a tagged recombinant protein. In this experiment, the FLAG-tagged Tar is expressed from an IPTG inducible plasmid.
2. Transfer 1 ml overnight *E. coli* culture to 100 ml 1% Tryptone medium and grow at 30 °C. This is the optimal growth condition for flagellar motility and Tar polar localization. Add 25 µM IPTG after 1 h to induce the target protein tagged with a FLAG epitope (Tar-FLAG). Grow the cells for additional 2-3 h.
3. Pellet the cells (6,000 x g for 5 min at room temperature). In this experiment, low temperature affects the normal polar localization of the Tar protein. Use room temperature in the following steps unless otherwise indicated. Resuspend the pellet in 15 ml PBS. Incubate at 30 °C with shaking for 1 h to allow newly synthesized Tar proteins localizing to poles.
4. Add 60 µl Halt protease inhibitor. Add 16 µg/ml RNase and 1.3 µg/ml DNase (final concentration). Disrupt the cells with an LV1 high shear fluid processor (Microfluidics) at 20,000 psi (the disruption takes only one sec). Cool the samples immediately on ice.
5. Spin (10,000 x g for 5 min at 4 °C) to remove unbroken cells.
6. Transfer 10 ml supernatant containing the membrane vesicles to a 15 ml tube. Add 2 ml PBST and mix by inverting the tube several times.
7. Add 150 µl anti-FLAG gel beads followed by incubation on ice for 1 h with slow shaking.
8. Transfer the mixture to an empty Poly-Prep chromatography column. Snap off the seal at the bottom of the column to allow the liquid to flow.
9. Rinse the beads twice with 8 ml cold PBST.
10. Cap the bottom of the column. Add 1 ml cold PBST to resuspend the beads. Transfer the mixture to a 1.5 ml tube (this step is important because proteins and vesicles are trapped non-specifically in the bed support layer at the bottom of the column).
11. Spin (6,000 x g for 1 min at 4 °C) to pellet the beads. Wash twice with 1 ml cold PBST.
12. Resuspend the beads in 85 µl 1% Triton X-100 in PBS and incubated at room temperature for 10 min to solubilize the inner membranes.
13. Centrifuge the mixture (6,000 x g for 1 min at room temperature). Transfer the supernatant containing the released proteins to a clean tube for further analysis.
14. Wash the pellet with 1 ml 1% Triton X-100 in PBS. Resuspend the pellet in 85 µl 1% SDS in water and incubate at 90 °C for 5 min to elute all other proteins, including the FLAG tagged protein. Centrifuge and collect the supernatant (6,000 x g for 1 min at room temperature).
15. Determine protein concentration in the sample using a Micro BCA protein assay kit.

Recipes

1. PBS
 - 40 mM sodium phosphate
 - 150 mM sodium chloride (pH 7.4)
2. PBST
 - PBS
 - 0.05% Tween 20

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References

1. Li, G. and Young, K. D. (2012). [Isolation and identification of new inner membrane-associated proteins that localize to cell poles in *Escherichia coli*](#). *Mol Microbiol* 84(2): 276-295.
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