Mar 22, 2022

A fluorescence-based in vitro scrambling assay for yeast MCP1 and human XK

Zhouping Hong[1], Karin Reinisch[1]

[1]Yale University

dx.doi.org/10.17504/protocols.io.kxygxzyqdv8j/v1

Reinisch lab

Zhouping Hong
Yale University

VPS13 proteins are proposed to function at contact sites between organelles as bridges for lipids to move directionally and in bulk between organellar membranes. VPS13s are found to interact with integral membrane proteins, like MCP1 in yeast or XK in humans. We showed that MCP1 and XK scramble phospholipids in vitro. Here I describe the detailed procedure of purification, reconstitution, and scrambling assay for both MCP1 and XK.

DOI

dx.doi.org/10.17504/protocols.io.kxygxzyqdv8j/v1

Zhouping Hong, Karin Reinisch 2022. A fluorescence-based in vitro scrambling assay for yeast MCP1 and human XK. **protocols.io** https://dx.doi.org/10.17504/protocols.io.kxygxzyqdv8j/v1

protocol ,

Mar 21, 2022

Mar 22, 2022

59708

:

Protein purification

1 Constructs encoding MCP1 (pCMV10-3xFLAG-Prs-MCP1) or XK (pCMV10-3xFLAG-Prs-XK) were transfected into Expi293 cells at the density of ~3 million/ml. The protein expression was enhanced with **[M]3.5 millimolar (mM)** valproic acid (VPA) 18 hours after transfection. The cells were harvested 48 hours after transfection and cell pellets were stored at -80°C.

2 MCP1 purification

2.1 The cell pellets were resuspended in buffer A (50 mM HEPES, pH7.0, 500 mM NaCl, 1 mM TCEP, 10% glycerol) containing 1× ☒cOmplete™, Mini, EDTA-free (Protease Inhibitor) **Roche Catalog ##11836170001)** and lysed using a Dounce homogenizer (15~20 passes).

The ratio of the volume of buffer A used to resuspend cell pellets to the volume of cell culture is 1:10. The amount of buffer A added here is important because it decides the amount of detergent you will use in step2.2, which needs to be enough to solubilize all membranes, but not too much to change the native state of the protein.

Citation: Zhouping Hong, Karin Reinisch A fluorescence-based in vitro scrambling assay for yeastÃÂ MCP1 and human XK https://dx.doi.org/10.17504/protocols.io.kxygxzyqdv8j/v1

2.2 To solubilize the protein, powdered DDM was added to the lysate at a final concentration of 1% w/v, and the lysate was gently agitated in the cold room for 90 minutes. Cell lysates were clarified via centrifugation at 100,000 g for 60 minutes and the supernatant was incubated with anti-FLAG M2 resin (Sigma Aldrich), which was pre-equilibrated with buffer B (buffer A, 0.02% DDM), at 4°C for 2 hours, and then the resin was washed with buffer B. To remove the chaperone, the resin was incubated with buffer B containing 5 mM MgCl2, 2.5 mM ATP containing buffer C at 4 °C overnight.

Calculation of the amount of resin needed: general yield of 3xFLAG-Prs-MCP1 from Expi293F cells is 40µg/100ml. The binding capacity of the resin is 0.6mg/ml settled resin and the slurry resin is in 50% solution. So you will need to use 134µl slurry resin to purify proteins from 100ml of Expi293 cells.

2.3 The next day, bound proteins were further washed with buffer B, then eluted using 0.25mg/ml 3xFlag peptide in Buffer B. The proteins were concentrated in a 10-kD molecular weight cutoff (MWCO) Amicon centrifugal filtration device (UFC501024) and quantified by Coomassie blue staining using BSA standards.

The elution was done 5 times, each time the same volume of elution buffer as the bed resin was added to the resin, incubated for 30 minutes, and the eluant was collected.

3 XK purification

3.1 The cell pellets were resuspended in buffer C (50 mM HEPES, pH 7.8, 200 mM NaCl, 1 mM TCEP, 10% Glycerol) containing 1× cOmplete EDTA-free protease inhibitor cocktail (Roche) and lysed using a Dounce homogenizer (15~20 passes).

The ratio of the volume of buffer A used to resuspend cell pellets to the volume of cell culture is 1:10. The amount of buffer A added here is important because it decides the amount of detergent you will use in step3.2, which

Citation: Zhouping Hong, Karin Reinisch A fluorescence-based in vitro scrambling assay for yeastÃÂ MCP1 and human XK
https://dx.doi.org/10.17504/protocols.io.kxygxzyqdv8j/v1

needs to be enough to solubilize all membranes, but not too much to change the native state of the protein.

3.2 To solubilize the protein, powdered GDN was added to the lysate at a final concentration of 1.5 % w/v, and the lysate was gently agitated in the cold room for 90 minutes. Cell lysates were clarified via centrifugation at 100,000 g for 60 minutes and the supernatant was incubated with anti-FLAG M2 resin (Sigma Aldrich), which was pre-equilibrated with buffer D (buffer C, 0.02% GDN), at 4°C for 2 hours, and then the resin was washed with buffer D. To remove the chaperone, the resin was incubated with buffer D containing 5 mM MgCl2, 2.5 mM ATP containing buffer C at 4 °C overnight.

Calculation of the amount of resin needed: general yield of 3xFLAG-Prs-XK from Expi293F cells is 90µg/100ml. The binding capacity of the resin is 0.6mg/ml settled resin and the slurry resin is in 50% solution. So you will need to use 300µl slurry resin to purify proteins from 100ml of Expi293 cells.

3.3 The next day, bound proteins were further washed with buffer D, then eluted using 0.25mg/ml 3xFlag peptide in Buffer D. The protein was gel filtrated with Superdex 200 increase 10/300 column (Cytiva) and concentrated in a 10-kD molecular weight cutoff (MWCO) Amicon centrifugal filtration device (UFC501024) and quantified by Coomassie blue staining using BSA standards.

The elution was done 5 times, each time the same volume of elution buffer as the bed resin was added to the resin, incubated for 30 minutes, and the eluant was collected.

Liposome preparation

4 ⊠16:0-18:1 PC (POPC) **Avanti Polar Lipids, Inc. Catalog #850457**, 9.5% POPE ⊠16:0-18:1 PE **Avanti Polar Lipids, Inc. Catalog #850757**, and 0.5% NBD-labeled lipid (NBD-PE, NBD-PC, or NBD-PS) in chloroform were mixed and dried under an N2 stream, and further vacuum dried for 30 minutes. The resulting lipid film was resuspended in buffer E (50 mM HEPES, pH 7.0, 200 mM NaCl) for MCP1 and in buffer F (50 mM HEPES, pH 8.0, 200 mM NaCl) for XK to generate a 10.5 mM lipid stock. The mixture was incubated at **37 °C** for 60 minutes, with

Citation: Zhouping Hong, Karin Reinisch A fluorescence-based in vitro scrambling assay for yeastÃÂ MCP1 and human XK https://dx.doi.org/10.17504/protocols.io.kxygxzyqdv8j/v1

vortexing every 10 minutes, and then freeze-thawed for ten cycles. Liposomes were extruded 31 times through a 400 nm polycarbonate filter and used within 6 hours.

It is important to use a 400 nm membrane to extrude liposomes used for membrane protein reconstitution.

No NBD-labeled lipids for leakiness control assay.

Proteoliposome preparation

5 Determine the optimal swelling conditions by a swelling titration assay

5.1 Wash the cuvette with miniQ water and buffer E or F.

5.2 Blank: set the parameter to OD540, set the blank point with your reconstitution buffer (buffer E or F).

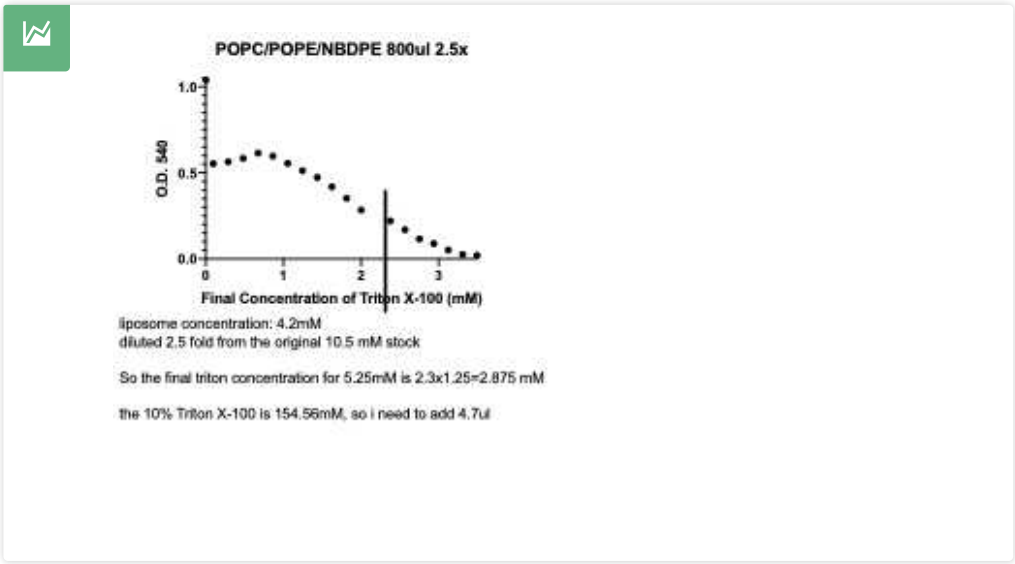
5.3 Dry the cuvette completely with an air duster.

5.4 Add 800µl liposome solution to the cuvette, record the OD540. If the readout is too high, like 2.0, dilute the liposome solution and read again, until the original read is ~1.

Citation: Zhouping Hong, Karin Reinisch A fluorescence-based in vitro scrambling assay for yeastÃÂ MCP1 and human XK https://dx.doi.org/10.17504/protocols.io.kxygxzyqdv8j/v1

5.5 Add 1µl of 10% Triton X-100 each time, and cap the cuvette, invert to mix for 30 seconds and record the OD540. repeat until the liposomes are completely dissolved.

5.6 Make a curve of the swelling titration assay. The typical curve with Triton X-100 looks like this, use the Triton X-100 concentration between saturation point and dissolving point (close to dissolving point, see curve below).



6 Prepare proteoliposomes: For each reaction, we have 250µl volume in total, the protein volume shouldn't be more than 10%, otherwise, the detergent in the protein sample could interfere with the reconstitution. You can have 125 µl liposomes in each reaction so the final concentration of lipids is 5.25mM, and 25µl protein sample, and the corresponding Triton X-100 amount (4.7µl), and fill the volume with your reconstitution buffer.

6.1 Swelling the liposomes: add the desired amount of Triton X-100 to 125µl liposomes, and add reconstitution buffer, agitate at room temperature for 2 hours. (add NBD-glucose to the NBD-glucose control group at this step)

6.2 Add the pre-purified protein into the solution, agitate for 1h at room temperature.

Citation: Zhouping Hong, Karin Reinisch A fluorescence-based in vitro scrambling assay for yeastÃÂ MCP1 and human XK
https://dx.doi.org/10.17504/protocols.io.kxygxzyqdv8j/v1

6.3 Prepare BioBeads: take some Biobeads into a 50ml tube, wash with methanol 3 times, miniQ water 2 times, reconstitution buffer once. For each wash, add liquid to the BioBeads to fill the tube, agitate at room temperature for 10min, discard the liquid. Pour the BioBeads to KimWIpe to dry it.

Calculate the amount of BioBeads you need: there are two standards: 1) from BioBeads manual: 20mg biobeads per 1.4mg Triton X-100 (1.5µmol) from the manual; 2) from the ATG9 paper: ~20mg of Biobeads SM-2 (Bio-Rad) per 0.3 µmol detergents. I usually take the average from these two. The total BioBeads amount I use is 4 times the average from the last step. Use ¼ for the first incubation, another ¼ for the second incubation, ½ for the last incubation.

6.4 Detergent removal: add ¼ of BioBeads to the solution, agitate at room temperature for 1h; add another ¼ of BioBeads to the solution, agitate at room temperature for 2h; transfer the solution to a new tube, add the last ½ of BioBeads, agitate at 4°C overnight.

6.5 The next day, transfer the solution into a new 1.5ml tube.

7 Determine the reconstitution efficiency: use Optiprep (Iodixanol) **Sigma Aldrich Catalog #D1556-250ML** as the gradient matrix

OptiPrep has lower viscosity as compared to polysucrose based products such as Histopaque or Ficoll. Besides, OptiPrep has a very large molecular weight and low ion strength, and it doesn't cause osmotic pressure to the vesicles. 15% OptiPrep is equal to 100mM glucose in terms of osmotic pressure. 60% OptiPrep density is 1.32g/ml.

7.1 Prepare different concentrations of OptiPrep using the reconstitution buffer. Each gradient needs at least 1ml. These will be the gradient solution

Keep the same concentration of NaCl in all gradients, because the contribution of ion strength from OptiPrep is very low, which we can ignore.

Citation: Zhouping Hong, Karin Reinisch A fluorescence-based in vitro scrambling assay for yeastÃÂ MCP1 and human XK https://dx.doi.org/10.17504/protocols.io.kxygxzyqdv8j/v1

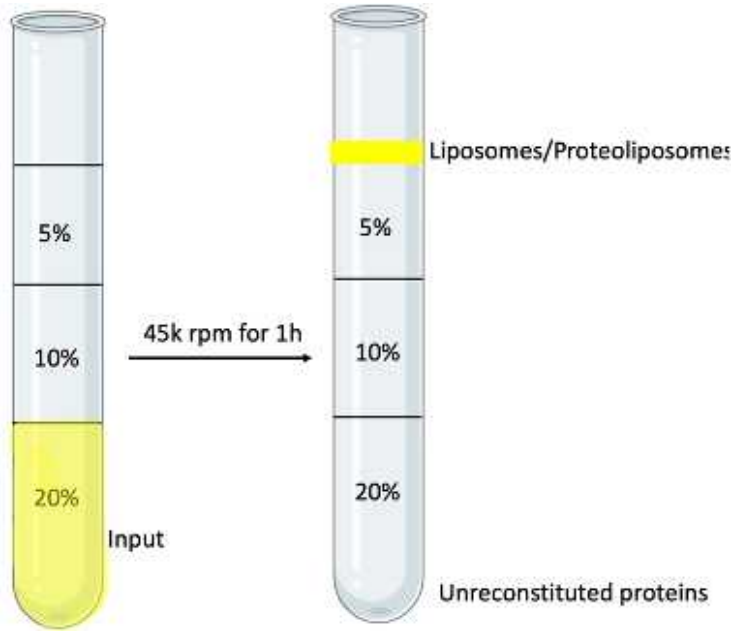
The final gradients are 5%, 10%, 20%, prepare 5%, 10%, 40% since you will need to mix the 40% OptiPrep with your sample to get the 20%.

7.2 Mix your sample with equal-volume 40% OptiPrep to get the 20% concentration gradient solution

7.3 Add the gradient solution into the ultracentrifuge tube: lot No: Z20214SCA (750µl)

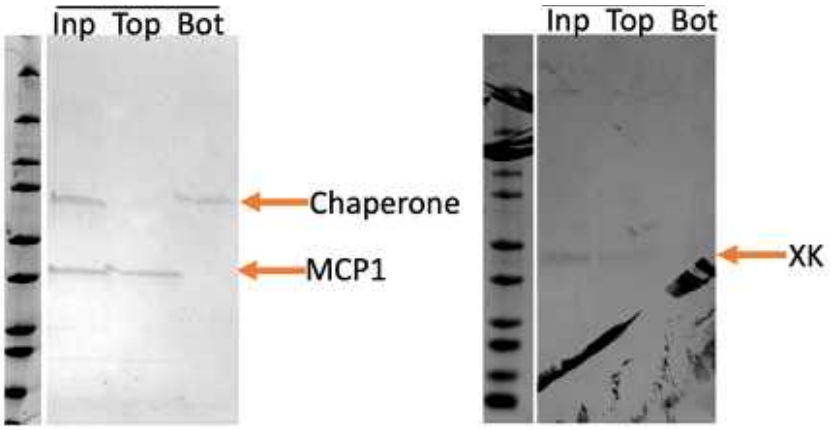
300 µl 20%; 200µl of the other gradients.
Use gel loading tips, slowly pipette down the solution to prevent disturbing the gradient.

7.4 Ultracentrifuge at 45,000 rpm 4°C for 1~1.5 hours, using SW 55 rotor. The protein itself goes to the bottom of the tube, and the proteoliposomes are at the very top. Separate the solution from the top, middle and bottom, run them on the SDS-PAGE gel.



The liposomes contain 0.5% NBD-labeled lipids, so that you can see which layer your liposomes are

Citation: Zhouping Hong, Karin Reinisch A fluorescence-based in vitro scrambling assay for yeastÃÂ MCP1 and human XK
https://dx.doi.org/10.17504/protocols.io.kxygxzyqdv8j/v1



MCP1 and XK are both in the top fraction, where liposomes are, suggesting a ~100% reconstitution efficiency

Scrambling assay

8 The scrambling assay was performed at 30°C in 96-well plates, with 100 µl reaction volumes of liposomes/proteoliposomes (~260 µM final lipid concentration) prepared as described above. To assess scrambling, NBD fluorescence after addition of dithionite (to 5 mM) was monitored (excitation at 460 nm, emission at 538 nm) using the Synergy H1 Hybrid Multi-Mode Reader (BioTek). Finally, additional dithionite (5 mM) and Triton X-100 (0.5%) were added. The Triton X-100 dissolves the liposomes, allowing complete quenching of the NBD.

8.1 Set the Synergy H1 plate reader temperature as **30 °C**

8.2 Add 95µl of the reconstitution buffer, 5µl of liposome/proteoliposome sample. Mix well.

8.3 Monitor the NBD fluorescence with excitation at 460 nm, emission at 538 nm for 5 minutes.

8.4 Prepare 100mM dithionite with 50mM Tris-HCl, pH 8.0 solution.

Citation: Zhouping Hong, Karin Reinisch A fluorescence-based in vitro scrambling assay for yeastÃÂ MCP1 and human XK
https://dx.doi.org/10.17504/protocols.io.kxygxzyqdv8j/v1

Dithionite is toxic and sensitive to light, and it can degrade very quickly, so prepare it right before use, and put it in the dark

- Weigh out a certain amount of dithionite, the MW of dithionite is 174.11g/mol, calculate the Tris-HCl you need to add to make it 100mM.
- Use 50mM Tris-HCl, pH8.0 to dissolve dithionite.
- For each sample, you will need to add 5µl 100mM dithionite, make sure the total amount of dithionite is enough for one measure

8.5 Add 5µl 100mM dithionite to each well, mix a little bit, monitor the emission changes for 30 minutes or 10 minutes.

8.6 Prepare another aliquot of dithionite. Add 5µl 10% Triton X-100 and 5µl 100mM dithionite to each well, mix a little bit. Monitor the changes for 5min, it should go to zero directly.

8.7 A similar protocol was used for the NBD-glucose leakiness control assay (Goren et al., 2014; Ploier and Menon, 2016), except that no NBD-lipids were incorporated into the liposomes or proteoliposomes. Instead, NBD-glucose (3 mM) was added during the destabilization step.

Citation: Zhouping Hong, Karin Reinisch A fluorescence-based in vitro scrambling assay for yeastÃÂ MCP1 and human XK https://dx.doi.org/10.17504/protocols.io.kxygxzyqdv8j/v1