Assembly of nanodiscs for use in cell-free expression using MSP1E3D1-His protein and DMPC phospholipids

Overview

This protocol describes the generation of fully assembled nanodiscs using membrane scaffold protein (MSP)1E3D1-His, the phospholipid Dimyristoyl-glycero-phosphocholine (DMPC) and the detergent sodium cholate. These nanodiscs can be used in cell-free expression reactions to directly integrate the nascent membrane protein into the nanodisc without any detergent added.

From 2 mg of MSP protein, this protocol yields about 50 µl nanodiscs in a concentration of about 10-15 mg/ml, corresponding to about 0.5-0.6 mM. We recommend to use an end concentration of 10-100 µM in an *E.coli* cell-free extract, depending on the membrane protein expression rate. This corresponds to 1-10 µl of a nanodisc solution concentrated to 0.5 mM in a total cell-free reaction volume of 50 µl. The MSP included in the assembly kit carries an N-terminal His tag and can therefore be purified using e.g. PureCube Ni-NTA Agarose or other IMAC matrices.

In our Nanodisc Assembly kit, the ratios of MSP:lipid have been optimized to 1:115. This ratio was described to be best suited for the generation of nanodiscs for cell-free expression using the MSP1E3D1 protein (3). Amounts of protein, lipid and cholate have been carefully aliquoted to yield this particular ratio. For the assembly of nanodiscs with other protein:lipid ratios, it is advisable to source protein and lipid separately. Please contact us for more information.

In this protocol, pre-aliquoted protein, lipid and cholate are mixed together. The detergent is slowly removed by dialysis, and MSP protein and phospholipids spontaneously assemble into nanodiscs. Size-exclusion chromatography separates fully assembled nanodiscs from unassembled protein and lipid to yield a homogeneous nanodisc fraction. This nanodisc fraction can be stored at -20°C for several months.

Please note that this protocol was optimized and significantly changed compared to previous versions. Please contact us at contact@cube-biotech.com if you have questions or need assistance optimizing a protocol for your application. All our protocols are available for free download at www.cube-biotech/protocols.

Equipment

- ☐ 37°C incubator (water bath or thermoshaker)
- ☐ Ice bath
- ☐ Micropipettor
- ☐ FPLC instrument (e.g. Äk.a or BioRad) with integrated UV detector and fraction collector
- ☐ Magnetic stirrer
- ☐ Centrifuge for 15 ml tubes (e.g. Falcon)
- ☐ Centrifuge for 1.5 ml tubes (e.g. Eppendorf)
- ☐ SDS PAGE equipment
- ☐ Optional: Western Blot equipment

Materials

- ☐ Cube Biotech Nanodisc Assembly Kit MSP1E3D1-His_DMPC, cat no. 26251. Kit contents: 2 mg MSP1E3D1-His protein, 5.09 mg DMPC and 20 mg sodium cholate
- ☐ Alternatively: 2 mg MSP1E3D1-His protein, (Cube Biotech cat.no. 26152), DMPC and sodium cholate from other sources
- ☐ EDTA 0.5 M pH 8.0 (e.g. Cube Biotech 61262)
- ☐ Sodium chloride (NaCl)
- ☐ Tris base
- ☐ Hydrochloric acid (HCl)
- ☐ Micropipetting tips
- ☐ Single use syringe (e.g. 1 mL)
- ☐ Single use needle (e.g. 0.45 x 25 mm)
- ☐ Dialysis tube (3-8 kDa cutoff)
- ☐ Protein concentrator (e.g. Amicon Ultra 10 kDa)
- ☐ Gel filtration column (e.g. HiLoad 16/600 or 10/300 Superdex 200 pg, GE Healthcare)
- ☐ Dithiothreitol (DTT)
- ☐ Glycerol
- ☐ Sodium dodecyl sulfate (SDS)
- ☐ Bromophenol blue
- ☐ Optional: PentaHis Antibody, (e.g. Cube Biotech cat.no. 40040)

Solutions and buffers

ND Buffer A (5 L)

Component	Final concentration	Molecular weight (g/mol)	Stock concentration	Amount needed for stock	Stock needed for buffer
NaCl	100 mM	58.44	5 M	146.1 g/ 500 mL	100 mL
Tris base, pH 7.4	20 mM	121.14	1 M	60.57 g/ 500 mL Set pH to 7.4 using HCl	100 mL
Instructions: Prepare two stock solutions and mix in the respective amounts to yield ND Buffer A. Tris stock solution can also be used to prepare ND Buffer B.					

ND Buffer B (50 mL)

Component	Final concentration	Molecular weight (g/mol)	Stock concentration	Amount needed for stock	Stock needed for buffer
Tris base, pH 7.4	20 mM	121.14	1 M	60.57 g/ 500 mL Set pH to 7.4 using HCl	1 mL
Instructions: Use stock solution prepared for Buffer A to make up Buffer B.					

5X SDS-PAGE Buffer, 10 mL

Component	Final concentration	Molecular weight (g/mol)	Stock concentration	Amount needed for stock	Stock needed for buffer
Tris-HCl, pH 6.8–7.0	300mM	121.14	1 M	121.14 g/ 1 L	3 mL
Glycerol	50% (v/v)	–	100% (v/v)	–	5 mL
SDS	5% (w/v)	–	–	–	0.5 g
Bromophenol blue	0.05% (w/v)	–	4%	–	125 µL
DTT	250 mM	154.25	1 M	1.54 g/ 10 mL	125 µL/aliquot
Instructions: Make sure to prepare a 1 M Tris-HCl stock by dissolving Tris base in 500 mL deionized water, adding HCl to a pH of 6.8–7.0, and adding water to a final volume of 1 L. For the SDS-PAGE Buffer, mix all components listed **except DTT** and add water to a total of 10 mL. Freeze 25 aliquots (375 µL each) at –20°C. Before use, add DTT to the needed single aliquots.					

Procedure

1. Prepare a 100 mM sodium cholate solution by adding 464 µl of ND Buffer B to the 20 mg aliquot (clear capped plastic vial).
2. Resuspend the contents of the white-marked brown glass vial containing 5.09 mg DMPC phospholipid with 150 µl of the 100 mM sodium cholate/ND Buffer B solution prepared in step 1. **Note:** Open the glass vial carefully as it contains a micro-glass tube held in position by a spring.
3. Incubate the solution obtained in step 2 for 30 min at 37°C on a thermoshaker, or alternatively mix by flicking/inverting the tube every 10 min.
4. Resuspend contents of the purple-capped plastic vial containing the lyophilized MSP1E3D1-His protein in 500 µl double distilled water.
5. Add 250 µl Buffer A and 250 µl of the 100 mM sodium cholate /ND Buffer B solution to the resuspended protein solution. Briefly spin the solution down. Keep on ice.
6. Add the entire volume of the solution obtained in step 3 containing DMPC and sodium cholate/ND Buffer B to the resuspended protein solution obtained in step 5.
7. Incubate the mix obtained in step 6 for 20 min at 4°C, then incubate for 20 min at 37°C.
8. Repeat step 7 twice, for a total incubation time of 2 h.
9. Fill the nanodisc mix into a dialysis tube of 3-8 kDa cutoff pore size and dialyse for two days at 4°C against ND Buffer A. Exchange the buffer about 2-4 times during this period.
10. Apply the nanodisc mix on a gel filtration column. Monitor absorbance at 280 nm.
11. Collect fractions of ca. 500 µL size, take an aliquot of 20 µL, add 5 µL of 5xSDS-PAGE buffer and analyze the samples by SDS PAGE. MSP proteins have an apparent molecular mass of around 20 kDa.
12. Concentrate the elution fractions which contain the nanodiscs using protein concentrators to 50 µl and store them at -20°C.

Note: The protein was lyophilized from a solution containing 4 mg/ml protein in ND Buffer A. Hence the final composition of the reconstituted protein is: 2 mg/ml MSP1E3D1-His, 20 mM Tris pH 7.4, 100 mM NaCl.

Note: During dialysis, the nanodiscs form, and sodium cholate is slowly removed from the solution.

Note: At 280 nm, aromatic residues in the protein are detected.

Note: Optionally, analysis of fractions in the western blot can be done e.g. with Penta His antibodies which recognize the his-tagged MSP1E3D1 protein.

Calculation to determine ratio of MSP and DMPC:

MW of MSP1E3D1, not counting the His tag: 30,620 Dalton or g/mol. MW of DMPC: 677.93 Da or g/mol
2 mg MSP1E3D1/ 30,620 g/mol = 0.065 µmol
To obtain a ratio of **1:115 (MSP:DMPC)**: 0.065 µmol x 115 = 7.51 µmol
7.51 µmol x 677.93 g/mol = 5.09 mg DMPC
5.09 mg DMPC is dissolved in 150 µL of 100 mM sodium cholate - Buffer B to yield a 50 mM lipid solution.

To obtain different ratios of MSP and DMPC, recalculate using the equation above, weigh required amount of DMPC manually and adjust volume of Buffer B.

References:
1. Bayburt, T. H., Grinkova, Y. V., & Sligar, S. G., 2002, Self-assembly of discoidal phospholipid bilayer nanoparticles with membrane scaffold proteins. Nanoletters, 2: 853-856.
2. Denisov, I. G., Grinkova, Y. V., Lazarides, A. A., & Sligar, S. G., 2004, Directed Self-Assembly of Monodisperse Phospholipid Bilayer Nanodiscs with Controlled Size. J. Am. Chem. Soc., 126: 3477-3487.
3. Roos, C., et al. 2014, High-level Cell-free production of membrane proteins with Nanodiscs. In: Alexandrov, K., and Johnston W.A. (eds) Cell-free protein synthesis: Methods and Protocols. Methods in Molecular Biology, vol. 1118, Springer Science+Business Media.