

Assembly of nanodiscs for use in cell-free expression using MSP1D1 Δ H5-His protein and POPC phospholipids

Overview

This protocol describes the generation of fully assembled nanodiscs using membrane scaffold protein (MSP)1D1 Δ H5-His, the phospholipid Palmitoyl-oleoyl-phosphatidylcholine (POPC) 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 μ l 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 μ M in an *E.coli* cell-free extract, depending on the membrane protein expression rate. This corresponds to 1-10 μ l of a nanodisc solution concentrated to 0.5 mM in a total cell-free reaction volume of 50 μ l. 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 1:40. This ratio was described to be best suited for the generation of nanodiscs for cell-free expression using the MSP1D1 Δ H5 protein. 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.com/protocols.

Equipment

- 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 MSP1D1 Δ H5-His_POPC, cat no. 26223. Kit contents: 2 mg MSP1D1 Δ H5-His protein, 3.12 mg POPC and 2 x 20 mg sodium cholate
- Alternatively: 2 mg MSP1D1 Δ H5-His protein, (Cube Biotech cat.no. 26122), POPC 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 (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

- Using one of the 20 mg sodium cholate aliquots (clear capped plastic vial), prepare a **200** mM sodium cholate solution by adding 232 μ l of ND Buffer B.
- Using the other 20 mg sodium cholate aliquot, prepare a **100** mM sodium cholate solution by adding 464 μ l of ND Buffer B.
- Resuspend the contents of the green-marked brown glass vial containing 3.12 mg POPC phospholipid with 82 μ l of the **200** 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.
- Incubate the solution obtained in step 3 for 30 min on ice. Mix by flicking or inverting the tube every 10 min.
- Resuspend contents of the green-capped plastic vial containing the lyophilized MSP1D1ΔH5-His protein in 500 μ l double distilled water.
- Add 250 μ l Buffer A and 250 μ l of the **100** mM sodium cholate /ND Buffer B solution to the resuspended protein solution. Briefly spin the solution down. Keep on ice.
- Add the entire volume of the solution obtained in step 4 containing POPC and sodium cholate/ND Buffer B to the resuspended protein solution obtained in step 6.
- Incubate the mix obtained in step 6 for 1 h on ice.
- 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.
- Apply the nanodisc mix on a gel filtration column. Monitor absorbance at 280 nm.
- Collect fractions of ca. 500 μ L size, take an aliquot of 20 μ L, add 5 μ L of 5xSDS-PAGE buffer and analyze the samples by SDS PAGE. MSP proteins have an apparent molecular mass of around 20 kDa.
- Concentrate the elution fractions which contain the nanodiscs using protein concentrators to 50 μ l and store them at -20°C.

Note: POPC is temperature-sensitive and should always be kept at 4°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 MSP1D1ΔH5-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 MSP protein.

Calculation to determine ratio of MSP and POPC:

MW of MSP1D1ΔH5, not counting the His tag: 19,488 Dalton or g/mol. MW of POPC: 760 Da or g/mol

2 mg MSP1D1/ 19,488 g/mol = 0.103 μ mol

To obtain a ratio of **1:40 (MSP:POPC)**: 0.103 μ mol x 40 = 4.11 μ mol

4.11 μ mol x 760 g/mol = 3.12 mg POPC

3.12 mg POPC is dissolved in 82 μ l of 200 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 POPC manually and adjust volume of Buffer B.

References:

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- 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.
- 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.