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We use this protocol and it's working

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Detection of accessible cholesterol in primary cilia using purified His-ALOD4-mNeon in 3T3 Fibroblasts

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ABSTRACT

There exist at least three different pools of cholesterol in the plasma membrane: the essential pool, the sphingomyelin-sequestered pool, and the accessible pool (Radhakrishnan et al, 2020). Ciliary Hedgehog signaling is regulated by the accessible pool of cholesterol (Kinnebrew et al, 2019), and His-mNeon-FLAG-ALOD4, a toxin-based probe, can be used to visualize this accessible pool. Here, we present a method for staining and measuring the amount of accessible cholesterol on cilia in 3T3 fibroblasts stably expressing Somatostatin Receptor (SSTR3)-mApple, a ciliary marker. The protocol described here is based upon previously established methods (Kinnebrew et al., 2019; Johnson and Radhakrishnan, 2021) and has also been used successfully to label cilia in RPE cells.

GUIDELINES

Purified ALOD4 is best used fresh and will lose activity if frozen; store at 4°C

MATERIALS

Purified His-tagged mNeon-FLAG-ALOD4 protein (prepared fresh as described here)
BL21 DE3 Rosetta plysS bacterial cells (Sigma # 70956-3)
1mL HiTrap Talon column (Cytiva #28953766)
PD-10 desalting column (Cytiva #17085101)
InstantBlue Coomassie Protein Stain (Abcam #ab119211)
His-tagged mNeon-FLAG-ALOD4 plasmid (Johnson and Radhakrishnan, 2021)
Biorad, 4–20% Mini-PROTEAN® TGX™ Precast Protein Gels, 12-well, 20 µl #4561095
NIH 3T3 cells (ThermoFischer #R76107)
Rat tail collagen (Gibco by Life Technologies # A10483-01)
Glass coverslips (Fisher Scientific #12-545-81)
Paraformaldehyde 16% Solution (Electron Microscopy Sciences # 15710) diluted fresh in 1X PBS

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His-mNeon ALOD4 purification

1 Make a 50 mL starter culture of BL21 DE3 Rosetta plysS cells expressing pRSET+ His-mNeon-FLAG-ALOD4, starting from a fresh colony picked from freshly transformed plates containing carbenicillin (100µg/ml) and chloramphenicol (34µg/ml). Grow Overnight with shaking in an Erlenmeyer flask at 37 °C.

2 Day 2. Using a 6 liter flask, prepare 2 X 2 liters LB by mixing 50 g LB powder with 2 L wate 20m Autoclave for 00:20:00 using a liquid cycle. Allow the media to cool to Room temperature

3 Add carbenicillin(100 µg /ml)and chloramphenicol (34 µg /ml) to each flask and shake at 37 °C until an OD 0.5-0.6 is reached. This usually takes ~2.5 hr.

4 Transfer flask to an 18 °C shaker and equilibrate temperature for 10-20 min.

5 Add IPTG to achieve a concentration of [M] 0.3 millimolar (mM)

6 Induce expression overnight with shaking at 180 rpm at 18 °C

7 Day 3. Transfer bacterial culture to 4, 1 L centrifuge bottles.

8 Pellet bacteria by spinning at 4000 RPM for 20 min 4°C; discard supernatant.

9 Add 1 Roche protease inhibitor tablet to 50 mL of lysis buffer, rotate at 4 °C to dissolve.

10 Set up a ~ 200 mL beaker with a stir bar at 4 °C in the cold room.

11 Resuspend pelleted bacteria On ice or in cold room using ~ 10 mL of lysis buffer for each bottle.

12 Vortex the bottles to help resuspend pellet; transfer resuspended pellets to the beaker with a stir bar.

13 Take 40 mL of this suspension to the Emulsiflex cell breakage device and have ready, 4 x 65ml polycarbonate ultracentrifuge tubes on ice for the homogenate from the next step. Pass 10 mL lysis buffer through the Emulsiflex once at 0-30K PSI to equilibrate the machine. Pass suspension through the Emulsiflex once at ~50K PSI to lyse cells. Collect directly into polycarbonate tubes On ice.

14 Balance tubes and then spin at 40,000 RPM for 00:45:00 in a 45Ti rotor at 4 °C to pellet ce 45m

debris. Collect supernatant and filter through a 500 mL, 0.45µm Millipore filter. Collect the filtered supernatant in a 100 mL glass beaker On ice.

15 Take a 1 mL HiTrap Talon column (Cytiva #28953766) and wash it with 10 column volumes (CV) of distilled, degassed water. Equilibrate column with 10CV lysis buffer (50mM HEPES pH 8, 500 mM NaCl, 5mM $MgCl_2$, 0.5mM TCEP, 10% glycerol, EDTA-free protease tablet)

16 Run the lysate through the column. Collect the flow through.

17 Wash the column with 30CV wash buffer (50mM HEPES pH 8, 500 mM NaCl, 5mM $MgCl_2$, 0.5mM TCEP, 10% glycerol, 20mM imidazole). Collect the wash fractions. Elute with an imidazole gradient of 100mM-500mM. Collect the elution fractions and analyze them by SDS PAGE.

18 Pool the fractions having the desired protein and buffer exchange the pool using a PD-10 desalting column (Cytiva #17085101) into the storage buffer (50mM HEPES pH 8, 150mM NaCl, 5mM $MgCl_2$, 0.5mM TCEP, 10% glycerol).

Note

ALOD4 will lose significant activity if frozen. Use fresh or store at 4°C and use within two weeks of purification

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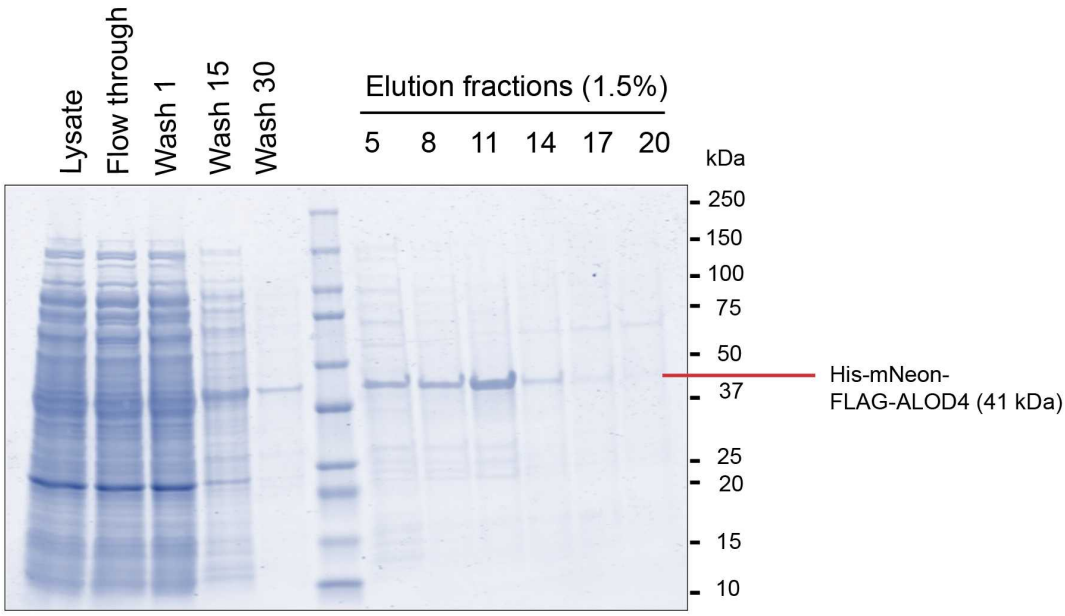

Purification of His mNeon FLAG ALOD4 using a Hi Trap Talon column (Affinity Chromatography)

Shown here is an example of SDS-PAGE analysis of the Hi Trap Talon purification. Samples were analyzed on 4–20% Precast Protein Gels. The yield is ~7mg from 4L culture. Depending on the purity of the protein after affinity chromatography, we either purify it further by size exclusion chromatography or more commonly, simply carry out buffer exchange using a PD-10 desalting column.

Cilia Staining

1d 1h 10m

20 Seed 0.3 X 10^6 cells onto collagen-coated coverslips. Once attached, starve the cells in media without serum or in low serum for 24:00:00 to induce ciliogenesis. 1d

21 Set up an incubation chamber for ALOD4-mNeon staining: place a metal tray (covered with a layer of parafilm) on ice for 20-30 minutes before paraformaldehyde (PFA) fixation.

22 Aspirate media and fix cells in 4% PFA in 1X PBS for 00:10:00 at Room temperature. 10m

23 Wash the cells three times in 1X PBS; transfer the coverslips to the incubation chamber and incubate 1h them for 01:00:00 in 4µM ALOD4-mNeon diluted in 1% BSA 1X PBS, sterilized by passage through a 0.2 µm filter.

24 Wash the cells twice in 1X PBS; fix the cells again in 2% PFA diluted in 1X PBS for 5 minutes On ice. Wash the cells again twice in 1X PBS.

25 Optional: stain with DAPI (1:1000) for 4 minutes on ice. Wash the cells twice in 1X PBS and mount them onto clean glass slides using 4 µl Mowiol. Air-dry the coverslips and proceed to imaging.

Example of ciliary accessible cholesterol staining

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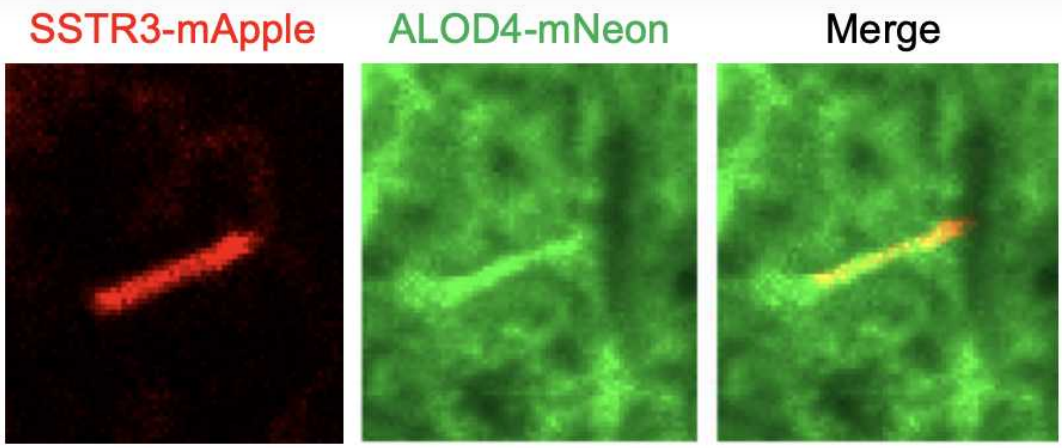

Shown here is an example 3T3 cell expressing SSTR3-mApple, starved for 24h to trigger ciliogenesis and stained with purified His-tagged mNeon-FLAG-ALOD4 protein according to this protocol.