Fractionation from Yao CK et al. (2017)

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Abstract

This protocol is from 'Flower Ca^{2+} channel in CME and ADBE' of Yao CK et al.

Please see the manuscript for the full method details.

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Guidelines

Primary antibody dilution used: Guinea pig anti-m/ratFwe2 (GP67), 1:500; Rabbit anti-SVP38, 1:1000 (Sigma); Rabbit anti-GM130, 1:5000 (Abcam); Mouse anti-Tubulin, 1: 10000 (Sigma); Mouse anti-α-Actin, 1:10000 (Sigma). Secondary HRP-conjugated antibodies were diluted in 1:5000 (Jackson ImmunoResearch).

Before start

You'll need:

RIPA buffer:

- 50mM Tris-HCl (pH 8)
- 150mM NaCl
- 1% NP-40
- 0.5% sodium deoxycholate
- 0.1%SDS

5 ml 0.32 M sucrose buffer:

- 320 mM Sucrose

- 1. 5 mM $MgCl_2$
- 1 mM EGTA
- 10 mM HEPES (pH 7.5)

Materials

- 50mM Tris-HCl (pH 8) by Contributed by users
- 150mM NaCl by Contributed by users
- 1% NP-40 by Contributed by users
- 0.5% sodium deoxycholate by Contributed by users
- 0.1%SDS by Contributed by users
- 1XSDS sample buffer by Contributed by users
- 320 mM Sucrose by Contributed by users
- 1. 5 mM MgCl2 by Contributed by users
- 1 mM EGTA by Contributed by users
- 10 mM HEPES (pH 7.5) by Contributed by users
- iced-cold Mini-Q water by Contributed by users
- 4mM HEPES by Contributed by users

Guinea pig anti-m/ratFwe2 (GP67), 1:500; Rabbit anti-SVP38, 1:1000 by Sigma

Rabbit anti-GM130, 1:5000 by Abcam

Mouse anti-Tubulin, 1: 10000 by Sigma

Diluted Secondary HRP-conjugated antibodies in 1:5000 by Jackson Immunoresearch

Protocol

Step 1.

To prepare subcellular fractions of adult rat brain, homogenize one adult brain (1 g) in 5 ml 0.32 M sucrose buffer (320 mM Sucrose, 1. 5 mM $MgCl_2$, 1 mM EGTA, 10 mM HEPES (pH 7.5)) using a Teflon glass homogenizer.

Step 2.

Centrifuge the homogenates (H) at 800 x g for 15 min at 4°C to yield pellets (P1) and supernatants (S1).

DURATION
00:15:00

Step 3.

Centrifuge S1 at 9,200 x g for 15 min at 4°C to obtain the pellets (P2) and supernatants (S2).

DURATION
00:15:00

Step 4.

Centrifuge supernatants (S2) at 100,000 x g for 2 h at 4°C to obtain the fractions of cytosol (S3) and light membrane (P3).

DURATION
02:00:00

Step 5.

Lyse the pellets (P2) in ice-cold Mini-Q water.

Step 6.

Equilibrate with 4 mM HEPES.

Step 7.

Mix for 30 min at 4°C.

DURATION
00:30:00

Step 8.

Centrifuge lysates at 25,000 x g for 20 min at 4°C to yield the crude synaptic vesicle fraction (LS1) and lysed synaptosomal membrane fraction (LP1).

DURATION
00:20:00

Step 9.

Centrifuge LS1 fraction further at 100,000 x g to obtain crude synaptic vesicles (LP2) and synaptosomal cytosol fraction (LS2).

Step 10.

Prepare a discontinuous sucrose gradient from 0.3-0.99 M by gradually layering the different concentrations of sucrose.

Step 11.

Load the S1 supernatants on sucrose gradient solution and centrifuged at 33,000 x g for 3 h at 4°C.

DURATION
03:00:00

Step 12.

Collect fractions from low- to high-density sucrose.

Step 13.

Boil these fractions in 1XSDS sample buffer and subjected to SDS-PAGE and western blotting.