Membrane and cytosol fractionation

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ABSTRACT

This protocol describes membrane and cytosol fractionation of cells expressing different DNAJC5 isoforms

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Protocol status: Working
We use this protocol and it's working

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Cytosol fractionation

1 Cells (one 10 cm dish) were cultured to 70% confluence and transfected with different constructs of DNAJC5

2 One day after transfection, we harvested the transfected cells by scraping in 1 ml B88 (20 mM HEPES-KOH, pH 7.2, 250 mM sorbitol, 150 mM potassium acetate, and 5 mM magnesium acetate) plus a cocktail of protease inhibitors

3 Cells were homogenized by 10 passages through a 22G needle

4 Homogenates were centrifuged at 500×g for 00:10:00 and the resulting post-nuclear supernatant (PNS) fractions were centrifuged at 100,000×g for 01:30:00 1h 40m

5 High-speed supernatant fractions were then subjected to a repeat centrifugation to achieve a clarified cytosol fraction

6 The pellet fraction was washed and resuspended in the same volume of B88

7 Resuspended material was also centrifuged again to collect a washed membrane fraction

8 Membranes were lysed in lysis buffer

Membrane fractionation

9 The PNS was subjected to differential centrifugation at 3000×g for 00:10:00 10m

10 The supernatant was centrifuged at 25,000×g for 00:20:00 20m

11 The supernatant was centrifuged at 100,000×g for 00:30:00 30m

12 Membrane fractions were normalized to phosphatidylcholine content and analyzed by immunoblot

Proteinase K protection assays 25m

13 The 25,000×g membrane fraction was aliquoted into three tubes: one without proteinase K, one with proteinase K (10 µg/ml), and one with proteinase K plus TritonX-100 (0.5%)

14 The incubation was conducted On ice for 00:20:00 20m

15 The reaction was stopped by sequential addition of PMSF (1 mM)

16 Add sample buffer. Then, samples were then heated on metal block at 95 °C for 00:05:00 and analyzed by SDS-PAGE and immunoblot. 5m