HHS Public Access

Author manuscript

Cold Spring Harb Protoc. Author manuscript; available in PMC 2016 August 24.

Published in final edited form as:

Cold Spring Harb Protoc. ; 2013(11): . doi:10.1101/pdb.prot073098.

Preparation of microsomes to study Ca^{2+} channels

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PROTOCOLS

Protocol 1

Preparation of rat cerebellar endoplasmic reticulum (ER) microsomes—The goal of this protocol is to prepare ER microsomes from native cerebellar tissue for planar lipid bilayer experiments. Prepared material is aliquited and stored at −80C freezer for many months prior to BLM experiments. Experiments with native cerebellar microsomes are used to study properties of native cerebellar $InsP_3R1$ and RyanR1 channels.

Materials—10–12 young (6–8 weeks old) rats

CO_2 inhalation station

Guillotine, surgical instruments

Ice backet

Teflon-glass homogenizers 55 ml, 30 ml, 2ml

Note: Quality of homogenizers is critical, especially for 2 ml homogenizer. Find most tightly fit 2 ml homogenizer. If not possible, use 10 ml homogenizer

J 25.50 Beckman rotor (or equivalent) and refrigerated centrifuge

Ti 50.2 Beckman rotor (or equivalent) and refrigerated ultracentrifuge

Liquid nitrogen

−80 freezer for storage

Method—Note: All manipulations are performed in the cold room. Cold solutions are used only. Rotors are kept in the cold room overnight to cool off.

1. 10–12 young rats (6–8 weeks old, weight 100–130 g) are killed by CO_2 inhalation for 1–2 min.
2. Cerebella are harvested by decapitation with a guillotine and placed in ice cold isotonic NaCl solution treated with PMSF from 500× stock of 5% in Ethanol (preferably fresh).

3. Cerebella are weighed (typically 0.23 g/cerebellum, with total wet weight of 2.8 g / 12 cerebella), minced and homogenized in 15 ml Homogenization Buffer (HB) in a 55 mL Glass-Teflon homogenizer deep on ice with stops.

   Note: HB needs to be pretreated with protease inhibitors before use.

   Note: Very important to keep the sample as cold as possible during homogenization procedure. Use hands and homogenize gently. Use frequent stops and keep on ice to cool off the solution. This is a criticial step. Overheating sample during homogenization is the most frequent cause of poor quality preparation.

4. Place homogenate in one 40 ml centrifugation tube for the J 25.50 Beckman rotor. Balance. Spin 15 min at 4,000 G (5750 rpm) at +4 C.

5. Transfer supernatant to one cold ultracentrifuge tube (30 ml) for Ti 50.2 Beckman rotor, filtering through cheese-cloth. Balance! Spin 30 min at ~ 90,000 G (30,000 rpm) and +4 C.

6. Remove supernatant. Resuspend the pelet with 1 ml High Salt Buffer (HSB) and and transfer to 30 ml Glass-Teflon manual homogenizer. Add HSB up to 15–20 mL. Perform 5–10 complete axial movements up and down.

   Note: Very important to keep the sample as cold as possible during homogenization procedure. Use hands and homogenize gently. Use frequent stops and keep on ice to cool off the solution.

7. Place homogenate to one 40 ml centrifugation tube for the J 25.50 Beckman rotor. Balance. Spin 15 min at 4,000 G (5750 rpm) and +4 C. This step removes any large pieces of poorly homogenized tissue that still remain in the sample.

8. Transfer supernatant to one cold ultracentrifuge tube (30 ml) for rotor Ti 50.2. Balance! Spin 30 min at ~ 90,000 G (30,000 rpm) and +4 C.

9. Remove supernatant. Resuspend pelets by adding 0.5 ml storage buffer (SB). Measure volume of suspension ($V_{initial}$) and calculate resulting sucrose concentration ($C_{initial}$ = 500 μl/$V_{initial}$) × 10%.

10. Transfer to 2 ml Glass-Teflon homogenizer

11. Add 50% sucrose from the stock to adjust the final sucrose concentration to exactly 10% using this formula to calculate volume of addition (V_{add}):

$$V_{add} = \frac{10\% - C_{initial}}{40\%} V_{initial}$$

    Note: It is critical to have final sucrose concentration at exactly 10%. Typcally $V_{initial}$ is about 800 μl, resulting in $C_{initial}$ = 6.25% and V_{add} = 75

µl. Final volume $V_{fin} = V_{initial} + V_{add}$ = 875 µl and final sucrose concentration C_{final} = (500 µl × 10% + 75 µl × 50%) / 875 µl = 10%.

12. Perform 5–10 axial movements to disperse the microsomes and the sucrose.

13. Aliquote 10–20 ul (as desired) to cold labeled microtubes and immediately freese by deeping into liquid Nitrogen. Transfer to −80 C for storage.

Note: The ER microsomes are stable at −80 C for months and even years. However, each fraction can be used only once in BLM experiments. No repetitive freeze-thaw.

14. The protein concentration in the final microsomal fraction (determined by the standard BioRad assay) should be in the range 4–5 mg/ml for this kind of MS preparations and the yield approximately 1 mg microsomal protein/g wet cerebellar tissue. The yield that is too high or too low indicate problems with execution of the procedure.

Buffer recipies for rat ER microsomal preparation: Homogenization Buffer (HB)

5 mM NaN_3

20 mM HEPES, pH 7.4 with KOH

High salt Buffer (HSB):

0.6 M KCl

5 mM NaN_3

20 mM $Na_4P_2O_7$

10 mM HEPES, pH 7.2 with HCl

Storage Buffer (SB):

10 % Sucrose

10 mM MOPS, pH 7.0 with KOH

Protease inhibitor stocks to be added to HB immediately before use—

	From stock	Final concentration
Aprotinine 1000×	1.9 mg/mL in water, +4 C	
Leupeptin 1000×	1.0 mg/mL in water, stored at − 20 C	2 uM
EDTA 100×	0.1 M	1 mM
Benzamidin 100×	20 mg/mL in water (FRESH!)	1 mM

	From stock	Final concentration
AEBSF 500×	25 mg/mL	
Pepstatin 500×	1 mg/ 0.2 mL in MetOH stable for one month at +4 C	
PMSF 500×	50 mg/mL in EtOH (FRESH!)	1 mM

Protocol 2

Preparation of Sf9 cells endoplasmic reticulum (ER) microsomes—The goal of this protocol is to prepare ER microsomes from Sf9 cells infected with $InsP_3R$ or RyanR recombinant baculoviruses. Prepared material is aliquited and stored at −80C freezer for many months prior to BLM experiments. Experiments with Sf9 ER microsomes are used to compare properties of different $InsP_3R$ and RyanR isoforms and to perform structure-function studies of $InsP_3Rs$ and RyanRs.

Materials—Sf9 cells suspension culture facilities

Sonicator

Ice backet

Glass-teflon homogenizers 55 ml, 30 ml, 2ml

Note: Quality of homogenizers is critical, especially for 2 ml homogenizer. Find most tightly fit 2 ml homogenizer. If not possible, use 10 ml homogenizer

J 25.50 Beckman rotor (or equivalent) and refrigerated centrifuge

Ti 50.2 Beckman rotor (or equivalent) and refrigerated ultracentrifuge

Liquid nitrogen

−80 freezer for storage

Method—Note: All manipulations are performed in the cold room. Cold solutions are used only. Rotors are kept in the cold room overnight to cool off.

1. Infect 150 ml of Sf9 cells in exponential growth phase with 15 ml of high-titer (P3 or P4) baculovirus encoding $InsP_3R$. Incubate in 27°C shaker.

   Note: Temperature control is critical. Keep temperature constant at 27°C

   Note: It is essential that Sf9 cells are in exponential growth phase. Use fresh batch of cells if expression is low.

2. Collect cells 48 h post-infection by centrifugation 5–7 min at 3000 rpm in Beckman rotor GH 3.8, +4°C. Remove supernatant.

3. Wash the cells with PBS, then resuspend cells in 25 mL of ice-cold Sf9 Homogenization Buffer (SF9HB) (with addition of protease inhibitors) in a 50 ml Falcon tube.

   Note: SF9HB needs to be pretreated with protease inhibitors before use.

4. Sonicate on ice with stops (at OC 4.5, DC 25) for 2 min.

   Note: Avoid excessive sonication and overheating. Sonicate with short bursts and pause between bursts to cool off the preparation. Keep on ice.

5. Homogenize sonicated cell mixture with 10 strokes of 55 ml Teflon/glass homogenizer for about 10 repetitions.

   Note: Very important to keep the sample as cold as possible during homogenization procedure. Use hands and homogenize gently. Use frequent stops and keep on ice to cool off the solution

5. Collect homogenate and place into one 40 ml centrifugation tube for the J 25.50 rotor.

6. Perform steps 4 – 14 from Protocol 1.

Buffer recipies for Sf9 ER microsomal preparation: Sf9 Homogenization Buffer (SF9HB)

0.5 M Sucrose

20 mM HEPES, pH 7.4 with KOH

High Salt Buffer (HSB), Storage Buffer (SB), protease inhibitor stock solutions as the same as in Protocol 1.