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Amplification of Purified Prions *In Vitro*

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Abstract

The infectious agents of prion diseases are unorthodox, and appear to be composed primarily of a misfolded glycoprotein called the prion protein (PrP). Replication of prion infectivity is associated with the conversion of PrP from its normal, cellular form (PrP^C) into a pathogenic form (PrP^{Sc}), which is characterized biochemically by relative detergent-insolubility and protease resistance. Several techniques have been developed, in which PrP^C molecules can be converted into the PrP^{Sc} conformation *in vitro* (1–8). These biochemical techniques recapitulate several specific aspects of *in vivo* prion propagation (1–3), and one method, the protein misfolding cyclic amplification (PMCA) technique, has also been shown to amplify infectivity (5).

In this chapter, we describe a method for amplifying PrP^{Sc} molecules from hamster prions *in vitro* using purified substrates. Specific protocols for substrate preparation, reaction mixture, and product detection are explained. Purified PrP^{Sc} amplification assays are currently being used to study the biochemical mechanism of prion formation.

Keywords

prion; PrP^{Sc}; purification; amplification; scrapie

1. Introduction

Mammalian prions are the infectious agents of transmissible neurodegenerative diseases affecting humans and other animals such as Creutzfeldt-Jakob Disease (CJD), bovine spongiform encephalopathy (BSE), chronic wasting disease (CWD), and scrapie (9). Unlike conventional infectious agents, prions lack informational nucleic acids, and therefore their mechanism of propagation has aroused great interest (10). The observation that transformation of a normal glycoprotein (PrP^C) to a misfolded isoform (PrP^{Sc}) accompanies disease progression led Prusiner to propose that self-induced protein conformational change is the mechanism of prion propagation (11).

Several laboratories have used a biochemical approach to study the mechanism of PrP^{Sc} formation. Caughey and colleagues first showed that purified PrP^C molecules could be converted into PrP^{Sc} molecules in a cell-free system (1). Furthermore, the specificity of the *in vitro* conversion process induced by template PrP^{Sc} molecules recapitulates the species and

strain specificity of prion transmission *in vivo* (1–3). Soto and colleagues showed that PrP^{Sc} molecules and prion infectivity could be amplified more efficiently by subjecting scrapie-infected and normal brain homogenates to the Protein Misfolding Cyclic Amplification (PMCA) technique (5), which involves cycles of sonication and incubation and generates amplification of PrP^{Sc} molecules in a manner analogous to the Polymerase Chain Reaction (PCR) for DNA molecules (4). Our laboratory has used biochemical purification and reconstitution techniques to show that (1) accessory polyanionic molecules facilitate efficient amplification of PrP^{Sc} *in vitro* (8), and (2) copper ions potently inhibit PrP^{Sc} formation (12). The ability to generate PrP^{Sc} molecules from purified and synthetic substrates provides a unique opportunity to study the composition and structure of prions.

2. Materials

2.1. Making the immunoaffinity column

1. 1 M RNase-free Tris pH 8.0 (Ambion, Austin, TX).
2. RNase-free 5 M NaCl (Ambion).
3. RNase-free 0.5 M ethylenediaminetetraacetic acid (EDTA) pH 8.0 (Ambion).
4. Phosphate buffered saline without calcium or magnesium (PBS) (Mediatech, Herndon, VA).
5. 200 mM triethanolamine pH 8.0 (Acros, Geel, Belgium).
6. 1 M 3-(N-Morpholino)-propanesulfonic acid (MOPS) (Sigma, St. Louis, MO) pH 8.0.
7. 0.2 M glycine pH 2.5 (Fisher Scientific, Pittsburg, PA).
8. PBS, 1% Triton X-100.
9. PBS, 1% Triton X-100, 20 mM Tris pH 8.0.
10. 20 mM MOPS pH 8.0, 0.25 M NaCl, 5 mM EDTA, 1% Triton X-100
11. 2% sodium azide (Sigma).
12. Protein A agarose 50% slurry (6–7 mg/ml resin binding capacity for mouse IgG) (Pierce, Rockford, IL, distributed by Fisher Scientific, catalog # PI20333).
13. Dimethyl pimelimidate × 2HCl (DMP) (Pierce, distributed by Fisher Scientific, catalog # PI21666).
14. 3F4 monoclonal antibody (1 mg/ml, Signet, Dedham, MA, catalog # 9620).
15. 1.5 cm I.D. Econo-Pac column (Bio-Rad, Hercules, CA, catalog # 7321010) with upper bed support.
16. 1.5 cm I.D. Econo-Pac column adapter (Bio-Rad catalog # 7380019).
17. End-over-end rotator.
18. Low speed centrifuge (Centra CL3R, with rotor 243, Thermo Electron Corporation, Waltham, MA).

2.2. Immunopurification of PrP^C

1. 3F4 immunoaffinity column (1 ml packed volume) fitted with column adapter, prepared as described above
2. Protein A agarose (Fisher catalog # PI20333) packed in a 1.5 cm I.D. Econo-Pac column (Bio-Rad catalog # 7321010) (1 ml packed volume) with upper bed support

(included). Use forceps and the top end of a 5 ml serological pipette to insert the upper bed support flush against the top of the resin. Be careful not to crush the resin, which would impede flow.

3. SP-Sepharose (Sigma catalog # S1799) ion exchange resin (1.5 ml ml packed volume) in a 1.5 cm I.D. Econo-Pac column (Bio-Rad catalog # 7321010) with upper bed support.
4. Zeba buffer-exchange columns (4 × 10 ml) (Pierce, distributed by Fisher Scientific, catalog #NC9286535).
5. 500 ml Stericup-GP filtration system (0.22 µm pore diameter), polyethersulfone, radio-sterilized (Millipore catalog # SCGPU05RE).
6. Low speed centrifuge (Centra CL3R, with rotor 243, Thermo Electron Corporation, Waltham, MA).
7. Ultracentrifuge (Avanti J30I with rotor JA-30.50Ti, Beckman Coulter, Fullerton, CA), with appropriate tubes.
8. Biohomogenizer Mixer (Biospec Products, Bartlesville, OK, distributed by Fisher Scientific, catalog # 15-338-51).
9. 50 ml Dounce homogenizer (Kontes, Vineland, NJ).
10. Peristaltic pump and 1.6 mm I.D. tubing.
11. Column stopcocks.
12. Molecular biology grade water (Ambion) to make solutions.
13. PBS.
14. 0.5 M MOPS stock solutions pH 7.0, 7.5, and 8.0.
15. 0.5 M 2-Morpholinoethanesulfonic acid (MES) (Sigma catalog # M5287) stock solution pH 6.4.
16. 10% Triton X-100, 10% sodium deoxycholate (DOC) (Sigma catalog # D6750).
17. Immunoaffinity equilibration buffer: PBS 1% Triton X-100, 1% DOC.
18. Immunoaffinity wash buffer 1: 20 mM MOPS pH 8.0, 0.5 M NaCl, 5 mM EDTA, 0.5% Triton X-100.
19. Immunoaffinity wash buffer 2: PBS, 0.5% Triton X-100.
20. 0.2 M glycine pH 2.5.
21. 1 M Tris pH 9.0.
22. SP equilibration buffer: 20 mM MES pH 6.4, 0.15 M NaCl, 0.5% Triton X-100.
23. SP wash buffer: 20 mM MOPS pH 7.0, 0.25 M NaCl, 0.5% Triton X-100.
24. SP elution buffer: 20 mM MOPS pH 7.0, 0.50 M NaCl, 0.5% Triton X-100.
25. Exchange buffer: 20 mM MOPS pH 7.5, 0.15 M NaCl, 0.5% Triton X-100.
26. PBS, 0.02% sodium azide.
27. Complete™ protease inhibitor cocktail tablets (containing EDTA) (Roche catalog # 1169498001).
28. Complete™ protease inhibitor cocktail tablets (without EDTA) (Roche catalog # 11873580001).

29. Ice in buckets.
30. Six Syrian hamsters ~8–12 weeks of age, either sex.
31. Dissecting tools to remove brains from skulls (Fisher Scientific: spatulas-catalog # 14-375-20; forceps-catalog # 13812-36; scissors-catalog # 13-808-4).

2.3. Preparation of PrP²⁷⁻³⁰ template

1. 10% (w/v) Sc237-infected hamster brain homogenate in PBS. The hamster should be in the terminal stages of disease, showing clinical signs of scrapie.
2. PBS, 1% Triton X-100.
3. 2 mg/ml proteinase K diluted from stock solution into water (~48 U/mg) (catalog # 03115828001, Roche).
4. 0.3 M (0.0522 g/ml) phenylmethylsulfonyl fluoride (PMSF) (Roche catalog # 11359061) in methanol (made immediately before use to avoid degradation).
5. 3000MPD sonicator with deep microplate horn (Misonix, Farmingdale, NY) with custom acrylic tube holder designed to keep a 1.5 ml microfuge tube ~ 1 cm from the center of the horn and ~3 mm off the surface of the horn.
6. Micro-ultracentrifuge (Discovery M120SE with rotor S45A, Sorvall, Hamburg, Germany) and 1.5 ml Safe-Lock® tubes (catalog # 2236320-4, Eppendorf, Westbury, NY).
7. Eppendorf Thermomixer (catalog #05-400-200, Brinkmann, Westbury, NY).
8. Cold water.
9. Ice in buckets.

2.4. In vitro PrP^{Sc} amplification

1. RNase-free TE Buffer pH 8.0 (Ambion).
2. Exchange buffer: 20 mM MOPS pH 7.5, 0.15 M NaCl, 0.5% Triton X-100.
3. PBS, 1% Triton X-100.
4. Sc237 PrP 27–30 diluted 1:10 in PBS, 1% Triton X-100
5. Purified hamster PrP^C preparation, see above.
6. Reaction buffer: 20 mM MOPS pH 7.0, 0.5 M imidazole (Sigma catalog # I-5513), 0.15 M NaCl, 50 mM EDTA. Use RNase-free stock solutions and water purchased from Ambion. Imidazole is a base, and should be neutralized before being added to the reaction buffer; we suggest preparing a stock solution of 1 M imidazole and titrating the pH to 7.0 using concentrated HCl.
7. Poly(A) RNA (polyadenylic acid potassium salt) (Sigma catalog # P9403) resuspended in RNase-free water at a concentration of 1–5 mg/ml (confirmed by A₂₆₀ measurements) and stored as aliquots in liquid nitrogen.
8. Eppendorf Thermomixer.
9. Microcentrifuge (model # 5417-C, Eppendorf).
10. Proteinase K.
11. SDS sample buffer, 2 × stock solution: 100 mM Tris pH 6.8, 4% Sodium Dodecyl Sulfate (SDS), 10% glycerol, 0.01% bromophenol blue, 10% β-mercaptoethanol.

12. Tube heating block set to 95°C.
13. Vortexer

2.5. SDS-polyacrylamide gel electrophoresis (SDS-PAGE) and Western transfer

1. Stacking gel mix: 0.6 M Tris pH 8.8, 0.16% SDS.
2. Resolving gel mix: 0.15 M Tris pH 6.8, 0.12% SDS.
3. Acrylamide solution: 40% Acrylamide/Bis solution 29:1 (Bio-Rad, catalog # 161–0147).
4. TEMED (Bio-Rad, catalog # 161–0801).
5. 10% ammonium persulfate in water.
6. Water-saturated butanol. Butanol is the top phase.
7. 10× Laemmli buffer (13): 60 gm Tris base, 288 gm glycine, 200 ml 10% SDS, make up to 2 L total volume with deionized water. The final pH should be 8.8
8. Anode buffer I: 300 mM Tris pH 10.4, 10% (v/v) methanol.
9. Anode buffer II: 25 mM Tris pH 10, 10% (v/v) methanol.
10. Cathode buffer: 25 mM Tris pH 9.4, 40 mM glycine, 10% (v/v) methanol.
11. Methanol.
12. Polyvinylidene fluoride (PVDF) membrane (Immobilon-P, Millipore, Billerica, MA).
13. Filter paper: 3mm, 15 cm × 17 cm (Whatman, Florham Park, NJ).
14. Electrophoresis apparatus: Hoefer SE 600 Ruby Vertical Standard (Amersham, Piscataway, NJ), with 18 × 16 cm glass plates and 1.5 mm spacers and 15-well comb.
15. Semi-dry transfer apparatus: Trans-Blot SD Semi-Dry Transfer Cell (Bio-Rad).
16. Power supply for electrophoresis and transfer apparatuses.
17. Pre-stained molecular weight markers: Precision Plus™ All Blue Protein Standard (Bio-Rad catalog # 161-0373) and PageRuler™ Prestained Protein Ladder (Fermentas, Hanover, MD, catalog # SM0671).
18. Pipette loading tips (USA Scientific Ocala, FL, catalog # 1022-0600).
19. Ruler
20. Scissors

2.6. Protein slot blot

1. Ethanol.
2. Methanol.
3. Immobilon-P polyvinylidene difluoride (PVDF) membrane.
4. Filter paper: 3mm, 15 cm × 17 cm (Whatman, Florham Park, NJ).
5. Minifold-2 slot blot apparatus (Schleisher and Schuell, Keene, NH, catalog # 10447800).
6. House vacuum attached to trap and regulated by adjustable valve.
7. Scissors.

8. Membrane-marking pen (Schleisher and Schuell catalog # 10499001).

2.7. Blot development and analysis

1. 3 M guanidine thiocyanate, 20 mM Tris pH 8.0.
2. 1 × TBST: 10 mM Tris pH 7.2, 0.15 M NaCl, 0.1% Tween-20 (made from 10 × TBST stock solution).
3. Non-fat milk (Hood, Chelsea, MA) in 1 × TBST (made by mixing 9 volumes milk with 1 volume 10 × TBST stock solution).
4. 3F4 monoclonal primary antibody (Signet).
5. Horseradish peroxidase (HRP) sheep anti-mouse IgG secondary antibody conjugate (Amersham, catalog # NA931)
6. Chemiluminescence reagent: Supersignal® West Pico Chemiluminescence Substrate (Pierce).
7. Saran wrap cling film.
8. Fujifilm LAS-3000 photodocumentation system attached to computer with Image Reader v2.0 and Image Gauge v4.22 software (Fujifilm USA, Valhalla, NY) installed.

3. Methods

3.1. Making the immunoaffinity column

1. Mix 2 ml of Protein A slurry with 8 ml (=8 mg) 3F4 antibody in a capped 15 ml polypropylene tube
2. Rotate the tube in an end-over-end rotator at ~6 r.p.m. for 30–60 min. at room temperature.
3. Centrifuge tube at $1,800 \times g$ for 2 min.
4. Remove supernatant and save. Optional: the supernatant should be depleted of 3F4 antibody, and this can be confirmed using an anti-mouse IgG antibody as a probe.
5. Wash the resin 2×12 ml 20 mM MOPS pH 8.0, 0.25 M NaCl, 5 mM EDTA, 1% Triton X-100. For this and all other resin washes described in this protocol, centrifuge at $1,800 \times g$ for 2 min to sediment the resin following each wash.
6. Wash the resin 2×12 ml 200 mM triethanolamine pH 8.0.
7. Add 5 ml of 10 mM DMP in 200 mM triethanolamine pH 8.0 (this solution should be made fresh, and unused DMP should be stored in a desiccated container in the dark at 4°C).
8. Rotate the tube in an end-over-end rotator at ~6 r.p.m. for 2 hrs at room temperature.
9. Quench cross-linking reaction by adding 50 μ l 1 M Tris pH 8.0.
10. Centrifuge tube at $1,800 \times g$ for 2 min and discard supernatant.
11. Wash the resin with 10 ml PBS, 1% Triton X-100, 20 mM Tris pH 8.0.
12. Wash the resin 2×10 ml with PBS
13. Wash the resin with 10 ml 0.2 M glycine pH 2.5
14. Wash the resin with 10 ml PBS, 1 % Triton X-100

15. Wash the resin 2×10 ml PBS
16. Resuspend the resin in 25 ml deionized water, and pack into the empty Econo-Pac column.
17. Attach the column adapter to the packed column, following the manufacturer's instructions.
18. Store the column in PBS 0.02% sodium azide at 4°C in between uses.

3.2. Immunopurification of PrP^C

1. Pre-chill all buffers, tubes, equipment, and rotors to 4°C. All steps of this protocol, except for brain dissection, should be performed at 4°C.
2. Attach stopcocks to all 3 columns, and a peristaltic pump to the immunoaffinity column.
3. Clean and equilibrate the protein A agarose pre-clearing column by sequentially passing through the following solutions: (1) 2 ml 0.2 M glycine pH 2.5; (2) 10 ml PBS; (3) 10 ml immunoaffinity equilibration buffer. Close stopcock when the fluid level is ~0.5 ml above the upper bed support.
4. Prime the peristaltic pump line attached to the 3F4 immunoaffinity column. Clean and equilibrate the immunoaffinity column by sequentially pumping through the following solutions at a flow rate of 0.75 ml/min: (1) 2 ml 0.2 M glycine pH 2.5; (2) 10 ml PBS; (3) 10 ml immunoaffinity equilibration buffer. Close stopcock when the fluid level is ~0.5 ml above the upper bed support.
5. Clean and equilibrate the SP-Sepharose ion-exchange column by sequentially passing through the following solutions: (1) 10 ml 2 M NaCl; (2) 25 ml water; (3) 10 ml SP equilibration buffer. Close stopcock when the fluid level is ~0.5 ml above the upper bed support.
6. Dissolve two CompleteTM protease inhibitor (+EDTA) cocktail tablets separately into 2×50 ml volumes of ice-cold PBS.
7. Euthanize 6 hamsters using carbon dioxide gas.
8. Rapidly collect brains using dissecting tools. Remove and discard dura mater, and quickly rinse blood off brains. The total time spent to collect tissue should not exceed 5 minutes.
9. Homogenize brains for 30–60 seconds in 40 ml total volume PBS plus protease inhibitors (+EDTA) using a Biohomogenizer Mixer set at 7,000 r.p.m. Keep the sample tube on ice during homogenization.
10. Centrifuge the sample at 3,200 r.p.m. for 20 minutes at 4°C, and discard the supernatant.
11. To the pellet, add 4 ml 10% Triton X-100, 10% DOC, plus PBS with plus protease inhibitors (+EDTA) to make a total volume of 40 ml. Resuspend the pellet using an ice-chilled Dounce homogenizer with pestle B (10 strokes).
12. Transfer the sample to an ultracentrifuge tube and incubate on ice for 30 min to solubilize membrane proteins.
13. Centrifuge at $100,000 \times g$ for 30 min at 4°C.
14. Filter the solubilized supernatant through 0.2 μ m Steri-cup to remove particulate matter. This step helps to prevent clogging of the chromatographic columns.

15. Pour the filtered sample over the pre-equilibrated, free gravity flow protein A agarose column, and collect the flow-through fraction.
16. Apply the protein A flow-through fraction to the immunoaffinity column at a flow rate of 0.75 ml/min.
17. Meanwhile, pre-equilibrate two 10 ml Zeba buffer exchange columns according to manufacturer's instructions with 3×5 ml SP equilibration buffer.
18. Wash the immunoaffinity column sequentially with 15 ml immunoaffinity wash buffer 1 and 10 ml of immunoaffinity wash buffer 2 at the same flow rate.
19. Disconnect the immunoaffinity column from the peristaltic pump, and manually elute column using a 10 c.c. syringe filled with 0.2 M Glycine pH 2.5. Elute at a flow rate of approximately 2–3 ml/min into the collection tube containing 0.8 ml 1 M Tris 9.0 until the total volume reaches 8 ml.
20. Mix contents of collection tube, and apply 4 ml sample to each pre-equilibrated Zeba buffer exchange column. Centrifuge according to manufacturer's instructions, collect the buffer-exchanged samples, and recombine.
21. Apply sample to pre-equilibrated, gravity flow SP-Sepharose column, using the stopcock to adjust the flow rate to ~0.75 ml/min.
22. Meanwhile, pre-equilibrate two 10 ml Zeba buffer exchange columns according to manufacturer's instructions with 4×5 ml exchange buffer plus Complete™ protease inhibitors (without EDTA).
23. Wash the SP-Sepharose column with 15 ml SP wash buffer.
24. Elute the SP-Sepharose column with 8 ml SP elution buffer.
25. Apply 4 ml eluate to each pre-equilibrated Zeba buffer exchange column. Centrifuge according to manufacturer's instructions, collect the buffer-exchanged samples, recombine, and freeze 0.5 ml aliquots at -70°C .

3.3. Preparation of PrP27-30 template

1. Mix 100 μl 10% scrapie brain homogenate, 895 μl PBS, 1% Triton X-100 and 5 μl 2 mg/ml proteinase K in a 1.5 ml micro-ultracentrifuge tube. Incubate the sample at 20°C in a Thermomixer shaking at 300 rpm for 30 min.
2. To stop the reaction, add 17 μl 0.3 M PMSF to the 1 ml sample (final PMSF concentration = 5 mM), and vortex.
3. Centrifuge at $100,000 \times g$ for 1 hr.
4. Carefully remove and discard the supernatant.
5. Add 200 μl ice-cold PBS, 1% Triton X-100 to the pellet.
6. Place sample in holder, and sonicate for 2×30 sec (with 1 min interval between bursts) using Misonix 3000MPD with output setting 6.0, and filled with 350 ml cold water.
7. Add 800 μl ice-cold PBS, 1% Triton X-100 to sonicated sample and vortex.
8. Centrifuge at $100,000 \times g$ for 30 min.
9. Repeat steps 4–8 two more times to wash the pellet thoroughly.
10. Vortex final sample and save 200 μl aliquots at -70°C .

3.4. In vitro PrP^{Sc} amplification

1. Make a working solution of 60 µg/ml poly(A) RNA in RNase-free TE buffer.
2. Prepare a 1:2 dilution (equal volume) of PrP^C substrate in exchange buffer.
3. Prepare a cocktail containing 50 µl of diluted PrP^C substrate, 10 µl reaction buffer, and 30 µl 60 µg/ml poly(A) RNA in TE, and 10 µl of PrP27-30 template. The final concentration of PrP^C in the cocktail is typically between 250–500 ng/ml.
4. Incubate components in Thermomixer for 16 hr at 37°C at 800 r.p.m. Freeze one sample at –70°C as a time zero control reaction to show the input PrP^{Sc} level.
5. Following incubation, briefly centrifuge samples to consolidate the condensed liquid.
6. Add proteinase K to each sample at a final concentration of 50 µg/ml and vortex. For comparison, it is useful to prepare an extra sample that is not subjected to protease digestion. This sample shows the input PrP^C level.
7. Incubate in Thermomixer for 1 hr at 37°C at 800 r.p.m.
8. Following incubation, add equal volume (100 µl) SDS loading buffer
9. Using a preheated, metal tube block, immediately heat samples at 95°C for 10 minutes.
10. Briefly centrifuge samples to consolidate the condensed liquid, and vortex.

3.5. SDS-polyacrylamide gel electrophoresis (SDS-PAGE) and Western transfer

1. Assemble gel cassette using 18 × 16 cm glass plates and 1.5 mm spacers. To avoid leaks, be sure that glass plates and spacers are completely flush at both the top and bottom of the cassette. To insure that the bottom of the cassette does not leak,
2. Prepare 12% gel by mixing 18.35 ml of resolving gel mix, 9 ml acrylamide solution, 2.5 ml ammonium persulfate solution, and 30 µl TEMED.
3. Quickly pipette the mixture into the gel cassette, leaving space for a stacking gel.
4. Carefully overlay with water-saturated butanol using a plastic sample pipette to a level ~2 mm above the top of the gel mixture. Avoid disrupting the top of the gel mixture when applying the butanol.
5. After the gel polymerizes, turn the cassette upside down over paper towels to drain the butanol. Check to insure that the top of the gel is level.
6. Place a 15-well comb into the top of the cassette.
7. Prepare the stacking gel by mixing 8.2 ml of stacking gel mix, 1 ml acrylamide solution, 1 ml 10% ammonium persulfate, and 13 µl TEMED.
8. Quickly pipette the mixture into the cassette, leaving ~5 mm space at the top of the cassette.
9. Prepare the running buffer by diluting 100 ml of the 10× Laemmli buffer with 900 ml of water in a measuring cylinder. Cover with Para-Film and invert to mix.
10. Once the stacking gel has set, carefully remove the comb and fill the wells with running buffer.
11. Load 70 µl of each sample per well. Load 7 µl pre-stained molecular weight markers.
12. Attach the upper chamber to the gel cassette, and add running buffer to the upper and lower electrophoresis chambers.

13. Connect the apparatus to a power supply. The gel can be run at 180V until the dye front reaches the bottom of the gel.
14. The samples that have been separated by SDS-PAGE are transferred to a charged, pre-equilibrated PVDF membrane using semi-dry electrophoretic transfer as follows.
15. The gel unit is disconnected from the power supply and disassembled.
16. Using a ruler, gel spacer, or glass plate, remove and discard the stacking gel.
17. Use a ruler to measure the height and length of the resolving gel in centimeters. Soak the resolving gel in cathode buffer for 15 min.
18. Charge an appropriately sized PVDF membrane in methanol for 2 sec, taking care not to wet the membrane prior to charging.
19. Rinse membrane in deionized water ~1–2 min.
20. Soak the PVDF membrane in anode buffer II for 15 min.
21. Carefully assemble the transfer sandwich as follows, using a serological pipette to ensure that no air bubbles are trapped in the sandwich: (1) anode electrode plate, (2) two sheets of filter paper wetted in anode buffer I, (3) one sheet of filter paper wetted in anode buffer II, (4) equilibrated PVDF membrane, (5) resolving gel, (6) three sheets of filter paper wetted in cathode buffer, (5) cathode electrode plate.
22. Finish assembling the trans-blot apparatus, connect to a power supply, and run on constant current at 2 mA/cm² gel surface area for 30 min.
23. Following electrophoretic transfer, the low and average molecular weight pre-stained markers should transfer completely. A small amount (up to 20%) of high molecular weight pre-stained markers may be retained in the gel.

3.6. Protein slot blot

1. Mix 50 µl each sample (previously boiled in SDS loading buffer) with 100 µl of 100% ethanol.
2. Charge a PVDF membrane in methanol for 2 seconds.
3. Equilibrate the PVDF membrane and 2 sheets of filter paper in water for ≥ 5 minutes.
4. Sandwich the water-equilibrated PVDF membrane backed by the 2 sheets of wet filter paper in the slot blot apparatus, following manufacturer's instructions. To insure a good seal, make sure that the membrane and filter paper do not overhang the slot blot gasket.
5. Apply samples to wells. Do not use corner wells.
6. Attach house vacuum gently (ideally, samples should take >1 minute to pass through the membrane).
7. Continue to run the vacuum for 30 seconds after the last sample passes through the membrane.
8. Disassemble the slot blot apparatus to recover the PVDF membrane. Use membrane marking pen to designate sample orientation. Proceed immediately to the blot development steps. Do not allow the membrane to dry out.

3.7. Blot development and analysis

1. Place the PVDF membrane (processed either by Western or slot blotting) in 3 M guanidine thiocyanate, 20 mM Tris pH 8.0 at room temperature for 30 min to denature PrP^{Sc} molecules completely.
2. Quickly rinse membrane 3 times with TBST
3. Block for 1 hr with buffered non-fat milk either at room temperature or 4°C.
4. Wash membrane 3 × 10 min in TBST either at room temperature or 4°C.
5. Incubate membrane overnight with 3F4 primary antibody diluted 1:5000 in TBST (final concentration 0.2 µg/ml) at 4°C.
6. Wash membrane 3 × 10 min in TBST at 4°C.
7. Incubate membrane 1 hr with HRP-labeled sheep anti-mouse IgG secondary antibody diluted 1:5000 in TBST (final concentration 0.1–0.2 µg/ml) at 4°C.
8. Wash membrane 3 × 10 min in TBST at 4°C.
9. Apply chemiluminescence substrate to membrane, according to manufacturer's instructions. Make sure that the entire membrane is covered with substrate.
10. Seal membrane in Saran Wrap to prevent evaporation.
11. Immediately capture chemiluminescent signal with LAS-3000 photodocumentation system according to the owner's manual instructions. Use the following settings on Image Reader version 2.0 software: standard sensitivity, filter = 1:through, iris = F0.8S, and incremental exposures at 30 sec intervals.
12. Save the longest exposure in which the signals are not saturated for quantitative analysis.
13. If desired, quantitate signals using Image Gague version 4.22 software according to the owner's manual instructions.

4. Notes

1. During the purification of PrP^C, clean each column as soon as possible after its use.
2. To clean the Protein A agarose column, wash sequentially with (1) 10 ml water to remove DOC, which precipitates under acidic conditions; (2) 8 ml 0.2 M glycine pH 2.5; (3) 15 ml water; (4) 15 ml PBS 0.02% sodium azide. Store in PBS, 0.02% sodium azide.
3. To clean the 3F4 immunoaffinity column, wash sequentially with (1) 15 ml water; (2) 15 ml PBS 0.02% sodium azide. Store in PBS, 0.02% sodium azide.
4. To clean the SP-Sepharose column, wash sequentially with (1) 8 ml 2 M NaCl; (2) 10 ml water; (3) 20 ml 0.02% sodium azide. Store in 0.02% sodium azide.
5. Store all columns at 4°C, and do not allow resins to dry out.
6. The expected yield of PrP^C from 6 hamster brains is ~16 µg PrP^C, and the expected concentration is ~2 µg/ml. If the concentration of PrP^C is higher or lower than expected, adjust the dilution of the substrate to be used in PrP^{Sc} amplification reactions accordingly.
7. The PrP^C purification protocol described here has only been used to purify hamster PrP^C, which is recognized by 3F4 antibody. Purification protocols for PrP^C molecules from other animal species would need to be developed empirically.

8. Handling of samples containing infectious prions should be carried out within a biosafety cabinet using BSL2 precautions. Infectious prion waste should be incinerated or inactivated by contact with 50% bleach for 1 hour.
9. The concentration of PrP²⁷⁻³⁰ molecules varies from preparation to preparation. For each preparation, we recommend testing a range of PrP²⁷⁻³⁰ dilutions in PBS, 1% Triton X-100 (up to 1:8) to determine empirically the optimal dilution for PrP^{Sc} amplification.

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Figure 1.

Silver stain analysis of purified PrP^C substrate. Twelve percent SDS-PAGE showing (from L to R): (1) Page-ruler prestained protein ladder (Fermentas, Hanover, MD catalog #SM0671) (estimated molecular weights = 170, 130, 100, 72, 55, 40, 33, 24, and 17 kDa); (2) eluate from 3F4 immunoaffinity column; (3) flow through fraction of SP-Sepharose column; and (4–7) successive 2 ml eluate samples from SP-Sepharose column. All chromatographic fractions (lanes 2–7) were concentrated prior to electrophoresis by the method of Wessel and Flugge (14)

Figure 2.

Western blot showing PrP^{Sc} amplification. Samples (from L to R): (1) reaction not subjected to proteinase K digestion, showing input PrP^C level; (2) time zero (frozen) reaction showing input PrP^{Sc} level; (3) amplification reaction without RNA; (4) amplification reaction with poly (A) RNA. Samples 2–4 were subjected to proteinase K digestion.