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Methods for Molecular Diagnosis of Human Prion Disease

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Summary

Human prion diseases are associated with a range of clinical presentations, and they are classified by both clinicopathological syndrome and etiology, with subclassification according to molecular criteria. Here, we describe updated procedures that are currently used within the MRC Prion Unit to determine a molecular diagnosis of human prion disease. Sequencing of the *PRNP* open reading frame to establish the presence of pathogenic mutations is described, together with detailed methods for immunoblot or immunohistochemical determination of the presence of abnormal prion protein in brain or peripheral tissues.

Key Words: Bovine spongiform encephalopathy; Creutzfeldt–Jakob disease; fatal familial insomnia; Gerstmann–Sträussler–Scheinker disease; kuru; prion; prion disease; prion protein; transmissible spongiform encephalopathy; variant Creutzfeldt–Jakob disease.

Introduction

Prion diseases are fatal neurodegenerative disorders that include Creutzfeldt–Jakob disease (CJD), Gerstmann–Sträussler–Scheinker disease (GSS), fatal familial insomnia (FFI), kuru, and variant CJD (vCJD) in humans ***(1-4)***. Their central feature is the posttranslational conversion of host-encoded, cellular prion protein (PrP^{C}), to an abnormal isoform, designated PrP^{Sc} ***(1, 5)***. Human prion diseases are biologically unique in that the disease process can be triggered through inherited germline mutations in the human prion protein gene (*PRNP*), infection (by inoculation, or in some cases by dietary

exposure) with prion-infected tissue or by rare sporadic events that generate PrP^{Sc} ***(1-3)***. Substantial evidence indicates that abnormal PrP isoforms are the principal, if not the sole, components of the transmissible infectious agent, or prion ***(1, 2, 5)***. The existence of multiple strains or isolates of prions, has been difficult to accommodate within a protein only model of prion propagation; however, considerable experimental evidence suggests that prion strain diversity is encoded within PrP itself and that phenotypic diversity in human prion diseases relates to differing physicochemical properties of abnormal PrP isoforms ***(5-12)***. Furthermore, the propagation of distinct abnormal PrP isoforms may be determined by the host genome ***(13-15)***.

Human prion diseases are associated with a range of clinical presentations, and they are classified by both clinicopathological syndrome and etiology, with subclassification according to molecular criteria ***(3, 4, 16-18)***. Approximately 85% of cases occur sporadically as Creutzfeldt–Jakob disease (sporadic CJD) at a rate of one to two cases per million population per year across the world, with an equal incidence in men and women ***(2, 19, 20)***. Approximately 15% of human prion disease is associated with autosomal dominant pathogenic mutations in *PRNP*, and to date more than 40 mutations have been described ***(2, 18, 21, 22)***. These include insertions of between four and twelve extra repeats within the octapeptide repeat region between codons 51 and 91, a two-octapeptide repeat deletion (OPRD), and various point mutations causing missense or stop substitutions **(Fig. 1)**.

Although human prion diseases are transmissible diseases, acquired forms have, until recently, been confined to rare and unusual situations. The most frequent causes of iatrogenic CJD occurring through medical procedures have arisen as a result of treatment with growth hormone derived from human cadavers or implantation of dura mater grafts ***(23-25)***. Less frequent incidences of iatrogenic human prion disease have resulted from transmission of CJD prions during corneal transplantation, contaminated electroencephalographic electrode implantation, and surgical operations using contaminated instruments or apparatus ***(25)***. The most well-known example of acquired prion disease in humans is kuru, which was transmitted by cannibalism among the Fore and neighboring linguistic groups of the Eastern Highlands in Papua New Guinea ***(17, 26-28)***. Remarkably, kuru demonstrates that incubation periods of infection with human prions can exceed 50 years ***(28)***. The appearance of vCJD in the United Kingdom from 1995 onward ***(29)***, and the experimental confirmation that this is caused by the same prion strain as that causing bovine spongiform encephalopathy (BSE) in cattle ***(7, 14, 30, 31)***, has led to widespread concern that exposure to the epidemic of BSE poses a distinct threat to public health in the United Kingdom and other countries ***(3, 32)***. The extremely prolonged and variable incubation periods of these diseases, particularly when crossing a transmission barrier ***(3, 33)***, means that it will be many years before the full consequences of human exposure to bovine prions can be predicted with confidence. In the meantime, significant numbers in the population may be incubating this disease ***(34)*** and pose a risk for transmitting infection to others via blood transfusion, blood products, tissue and organ transplantation, and other iatrogenic routes ***(32, 35-39)***.

Polymorphism at codon 129 of *PRNP* (**Fig. 2**) (encoding either methionine [M] or valine [V]) powerfully affects susceptibility to human prion diseases ***(20, 27, 40-44)***. About 38% of northern Europeans are homozygous for the more frequent methionine allele, 51% are heterozygous, and 11% homozygous for valine. Homozygosity at *PRNP* codon 129 predisposes to the development of sporadic and acquired CJD ***(20, 27, 40-43)***, and is most strikingly observed in vCJD where all pathologically proven cases studied so far have been homozygous for codon 129 methionine of *PRNP* ***(3, 38, 44-46)***. One heterozygous patient thought clinically to have vCJD did not have an autopsy examination ***(47)***. Heterozygosity at codon 129 is thought to confer resistance to prion disease by inhibiting homologous protein-protein interactions essential for efficient prion replication while the presence of methionine or valine at residue 129 controls the propagation of distinct human prion strains via conformational selection ***(3, 5, 32, 45, 46)***.

The clinical presentation of human prion disease varies enormously, and there is considerable overlap observed between individuals with different disease etiologies ***(3, 19, 22)*** and even in family members with the same pathogenic *PRNP* mutation ***(21, 22, 48-52)***. Progressive dementia, cerebellar ataxia, pyramidal signs, chorea, myoclonus, extrapyramidal features, pseudobulbar signs, seizures, and amyotrophic features can be seen in variable combinations. Criteria used for diagnosis of human prion disease are available ***(3, 53)***, and definite diagnosis of sporadic and acquired prion disease relies upon neuropathological examination and the demonstration of abnormal PrP deposition in the central nervous system by either immunoblotting or immunohistochemistry. In the

appropriate clinical setting , identification of a pathogenic *PRNP* mutation provides clear diagnosis of inherited prion disease and subclassification according to mutation; *PRNP* analysis is also used for presymptomatic genetic testing in affected families ***(3, 17, 18, 22, 48, 54, 55)***. Because of the extensive phenotypic variability seen in inherited prion disease and its ability to mimic other neurodegenerative conditions, notably Alzheimer's disease, *PRNP* analysis should be considered in all patients with undiagnosed dementing and ataxic disorders ***(3, 22, 49)***. In addition, we have recently described a novel clinical and pathological phenotype associated with a Y163X mutation in *PRNP*, a prion protein systemic amyloidosis, characterised by slow disease progression, diarrhoea, autonomic failure and neuropathy ***(56)***. This newly recognised novel disease phenotype indicates that *PRNP* analysis should also be considered in the investigation of unexplained chronic diarrhoea associated with a neuropathy or an unexplained syndrome similar to familial amyloid polyneuropathy.

Clinicians are fortunate that a range of sensitive and specific investigations are available to secure a diagnosis in life. Principal amongst these is magnetic resonance imaging (MRI) of the brain which shows increased signal on T2 or diffusion weighted images in the cerebral cortex, basal ganglia and thalamus in >80% of patients with sporadic CJD ***(57, 58)***, although these are sometimes overlooked ***(59)***. Cerebrospinal fluid (CSF) analysis is also helpful for differential diagnosis as the absence of a raised cell count or oligoclonal bands are reassuring from the point of view of inflammatory aetiologies. Raised CSF proteins 14-3-3, S100b and NSE are also found in ~90% sporadic CJD

patients although their presence is not specific for the disease. CSF PrP amplification technologies show promise as specific and sensitive confirmatory tests ***(60, 61)***.

The brains of patients with prion disease frequently show no recognizable abnormalities on gross examination at necropsy; however, microscopic examination of the brain at either necropsy or in ante-mortem biopsy specimens typically reveals characteristic histopathologic changes, consisting of neuronal vacuolation and degeneration, which gives the cerebral gray matter a microvacuolated or "spongiform" appearance accompanied by a reactive proliferation of astroglial cells ***(62-64)***. Although spongiform degeneration is frequently detected, it is not an obligatory neuropathologic feature of prion disease; astrocytic gliosis, although not specific to the prion diseases, is more constantly seen. The lack of a lymphocytic inflammatory response is also an important characteristic. Demonstration of abnormal PrP immunoreactivity, or more specifically biochemical detection of PrP^{Sc} in brain material by immunoblotting techniques is diagnostic of prion disease and some forms of prion disease are characterized by deposition of amyloid plaques composed of insoluble aggregates of PrP ***(62-65)***. Amyloid plaques are a notable feature of kuru and GSS ***(62, 65, 66)***, but they are less frequently found in the brains of patients with sporadic CJD, which typically show a diffuse pattern of abnormal PrP deposition ***(9, 62, 65)***. The histopathological features of vCJD are remarkably consistent and distinguish it from other human prion diseases, with large numbers of PrP-positive amyloid plaques that differ in morphology from the plaques seen in kuru and GSS in that the surrounding tissue takes on a microvacuolated appearance, giving the plaques a florid appearance ***(29, 67)***. The tissue distribution of PrP^{Sc} in vCJD

differs strikingly from that in classical CJD with uniform involvement of lymphoreticular tissues ***(35, 68-72)***. Depending upon the density of lymphoid follicles, PrP^{Sc} concentrations in vCJD peripheral tissues can vary enormously, with levels relative to brain as high as 10% in tonsil ***(35, 68)*** or as low as 0.002% in rectum ***(35, 39)***. Tonsil biopsy is used for diagnosis of vCJD, and to date it has shown 100% sensitivity and specificity for diagnosis of vCJD at an early clinical stage ***(3, 35, 38, 68)***, although some patients show scanty deposition of abnormal PrP and a large number of follicles may have to be examined by immunohistochemistry ***(73)***.

In this chapter, we update our previous contribution to Prion Protein Protocols ***(74)*** and describe the procedures that are currently used within the MRC Prion Unit to provide a molecular diagnosis of human prion disease. Methods for sequencing the *PRNP* open reading frame to establish the presence of pathogenic mutations and to determine *PRNP* polymorphic codon 129 genotype are described together with procedures used for immunoblot or immunohistochemical determination of the presence of abnormal PrP in brain or peripheral tissues.

2. Materials

2.1. Molecular Genetics

1. BACC3 DNA extraction kit from GE Healthcare Life Sciences.

2. TE buffer: 10 mM Tris and 1 mM EDTA, pH 8.0.

3. MegaMix Royal (Microzone, Haywards Heath, UK).

4. HyperLadder 100bp (Bioline, London, UK).

5. MicroClean (Microzone).

6. BigDye version 1.1 Cycle Sequencing kit (Applied Biosystems, Foster City, CA).

7. BetterBuffer (Microzone).

8. 0.5 M EDTA, pH 8.0, diluted fourfold in water.

9. Hi-Di formamide (Applied Biosystems).

10. Performance-optimized polymer 7 (Applied Biosystems).

11. DdeI restriction endonuclease, including NEBuffer 3 (New England Biolabs, Ipswich, MA).

12. MetaSieve agarose (Flowgen, Ashby, Leicestershire, UK).

13. PflFI restriction endonuclease, including NEBuffer 4 (New England Biolabs).

14. BsaI restriction endonuclease, including NEBuffer 3 (New England Biolabs).

15. TOPO TA cloning kit for sequencing (Invitrogen, Paisley, UK).

16. Luria-Bertani (LB) broth.

17. TaqMan GTXpress Master Mix (Life Technologies, Paisley, UK).

18 TaqMan MGB probes (Life Technologies, Paisley, UK).

2.2. Immunoblotting

1. Dulbecco's sterile phosphate-buffered saline (PBS) lacking Ca^{2+} and Mg^{2+} ions.

2. Duall tissue grinders (Anachem Ltd., Luton, Bedfordshire, UK).

3. Proteinase K (specific enzymatic activity ~30 Anson units/g) prepared as a stock solution of 1 mg/ml in water.

4. Sodium dodecyl sulfate (SDS) sample buffer.

a. A stock concentrate of 2× SDS sample buffer [142 mM Tris, 22.72% (v/v) glycerol, 4.54% (w/v) SDS, and 0.022% (w/v) bromphenol blue] is prepared in water and titrated to pH 6.8 with HCl.
b. This solution requires adjustment with reducing agent and proteinase K inhibitor immediately before use to produce 2× working SDS sample buffer.
c. For preparation of 0.5 ml of 2× working SDS sample buffer, mix the following: 440 µl of stock concentrate of 2× SDS sample buffer plus 20 µl of 2-mercaptoethanol plus 40 µl of 100 mM 4-(2-aminoethyl)-benzene sulfonyl fluoride prepared in water.
d. This produces 2× working SDS sample buffer of the following final composition: 125 mM Tris-HCl, 20% (v/v) glycerol, pH 6.8, containing 4% (w/v) SDS, 4% (v/v) 2-mercaptoethanol, 8 mM 4-(2-aminoethyl)benzenesulfonyl fluoride, and 0.02% (w/v) bromphenol blue.

5. Novex® 16% Tris-glycine SDS-polyacrylamide gel electrophoresis (PAGE) gels (Life Technologies Ltd, Paisley, UK).

6. Seeblue prestained molecular mass markers (Life Technologies Ltd).

7. SDS-PAGE electrophoresis buffer: 100 ml of 10× Tris-glycine, SDS concentrate [0.25 M Tris, 1.92 M glycine, 1% (w/v) SDS (National Diagnostics, USA)] plus 900 ml water.

8. Immobilon P transfer membrane.

9. Electroblotting buffer: 100 ml of 10× Tris-glycine concentrate [0.25 M Tris and 1.92 M glycine (National Diagnostics, USA)], 700 ml of water, and 200 ml of methanol.

10. PBST: 100 ml of 10× PBS concentrate (low in phosphate) (VWR, Lutterworth, UK), 900 ml of water, and 0.5 ml of Tween-20.

11. Anti-PrP monoclonal antibody 3F4 (Covance, Princeton, New Jersey).

12. Goat anti-mouse IgG (fab-specific) alkaline phosphatase conjugate (absorbed with human serum proteins) (Sigma-Aldrich Poole, Dorset, UK).

13. CDP-star chemiluminescent substrate (Life Technologies Ltd).

14. Carestream Biomax MR film (Anachem Ltd).

15. AttoPhos chemifluorescent substrate (Promega, Madison, WI).

    a. Mix 36 mg of Attophos substrate in 60 ml of Attophos buffer.

    b. Store as 3 ml aliquots at –20°C.

16. Sodium lauroylsarcosine (Merk Chemicals Ltd, Nottingham, UK).

17. Benzonase (Benzon nuclease purity 1 [25 U/µl], Merck Chemicals Ltd).

18. Sodium phosphotungstic acid stock solution.

    a. Stock solution is 4% (w/v) sodium phosphotungstic acid containing 170 mM $MgCl_2$ prepared in water, pH 7.4.

    b. For preparation of 10 ml of stock solution, add 0.4 g of sodium phosphotungstic acid and 0.35 g $MgCl_2 \cdot 6H_2O$ to a 50 ml polypropylene tube and make to ~9 ml with water.

    c. The pH of this solution is acidic and needs to be titrated with 5 M NaOH to pH 7.4 before adjusting to a final volume of 10 ml with water.

d. On addition of NaOH, immediate formation of insoluble $MgOH_2$ occurs that will redissolve on vortexing.

e. Addition of 5 M NaOH followed by vortexing and measurement of pH needs to be done repetitively.

f. For 10 ml of stock solution, addition of 360 μl of 5 M NaOH will generate pH 7.4.

2.3. Immunohistochemistry

2.3.1. Procurement

1. 10% buffered formal-saline.

2. Biopsy cassettes (R. A. Lamb, Eastbourne, UK).

2.3.2. Prion Deactivation with Formic Acid

1. Biopsy cassettes (R. A. Lamb).

2. 98% formic acid.

3. 2 M sodium hydroxide: 80 g of sodium hydroxide pellets in water to 1 liter.

4. 10% buffered formal-saline.

2.3.3. Tissue Processing

1. 10% buffered formal-saline.

2. Industrial methylated spirits (J.M. Loveridge Ltd., Southampton, UK), diluted in water to desired concentration.

3. Xylene.

4. Pure paraffin wax (R A Lamb).

2.3.4. Tissue Sectioning

1. Microtome (Leica, Wetzlar, Germany).

2. SuperFrost microscope slides (VWR, West Chester, PA).

2.3.5. Tissue Staining

1. Xylene.

2. Absolute ethanol diluted in water to desired concentration.

3. Harris hematoxylin (BDH).

4. Acid alcohol: 1% HCl in absolute ethanol.

5. Eosin Y solution 0.5%, aqueous (VWR).

6. Pertex mounting medium (Cox Scientific Ltd, Kettering, UK.).

7. Benchmark staining machine (Ventana Medical Systems, Illkirch CEDEX, France).

8. Protease 1 (Ventana Medical Systems).

9. Rabbit anti-glial fibrillary protein (Dako UK Ltd., Ely, Cambridgeshire, UK); antibody diluent (Ventana Medical Systems).

10. IViewDAB detection kit (Ventana Medical Systems), containing an inhibitor solution (3% hydrogen peroxide) (4 min), universal biotinylated secondary antibody (10 min), streptavidin-horseradish peroxidase solution (10 min), 3,3-diaminobenzidine and hydrogen peroxide (20 min), copper solution (4 min).

11. Hematoxylin (Ventana Medical Systems).

12. Bluing reagent (Ventana Medical Systems).

13. Tris-EDTA-citrate buffer, pH 7.8: 2.1 mM Tris, 1.3 mM EDTA, and 1.1 mM sodium citrate.

14. 98% formic acid.

15. 10 mM sodium citrate buffer, pH 6.0: solution A: 10.5 g of citric acid in 500 ml of deionized water and solution B: 29.41 g of sodium citrate in 1000 ml of water.

    a. Add 18 ml of solution A to 82 ml of solution B, and then adjust to 1 liter final volume with water.

16. Anti-PrP monoclonal antibody ICSM35 (D-Gen Ltd., London, UK).

17. Protease 3 (Ventana Medical Systems).

18. Superblock (Pierce Chemical, Rockford, IL).

19. Prepared with methanol GPR and 30% hydrogen peroxide.

20. Tris-buffered saline (TBS): 50 mM Tris, 145 mM NaCl, pH 7.6.

    a. For 1 liter of 10× stock, add 60.5 g of Trizma base and 84.7 g of sodium chloride to 800 ml of water.

    b. Adjust to pH 7.6 with 32.00 ml of concentrated HCl.

    c. Make to 1 liter final volume with water.

    d. Dilute 10× TBS to 1× concentration with water.

21. 4 M guanidine thiocyanate.

    a. Add 472.64 g guanidine thiocyanate in water to a final volume of 1 liter.

22. Normal rabbit serum (Dako UK Ltd.).

23. Biotinylated rabbit anti-mouse immunoglobulins (Dako UK Ltd.).

24. Strept ABComplex/HRP Duet kit (Dako UK Ltd.).

25. 3,3-diaminobenzidine tetrachloride.

a. Use at a final concentration of 25 mg in 100 ml of 1× TBS.

3. Methods

3.1. Molecular Genetics

3.1.1. Isolation of Genomic DNA from Blood

1. All procedures are performed within a class 1 microbiological safety cabinet situated within an ACDP level II containment laboratory with strict adherence to local rules of safe working practice. Informed consent for the analysis of a sample must be established before investigation. This may be obtained from the patient (or next of kin or other advocate in accordance with the Mental Capacity Act 2005 in the UK). For predictive testing, we expect evidence of appropriate genetic counselling before analysis.
2. Genomic DNA is extracted from whole anticoagulated blood (typically from a 5-ml EDTA tube) by using the Nucleon BACC3 DNA extraction kit (*see* **Subheading 2.1.**, **item 1**) following the manufacturer's instructions. DNA concentrations are determined using a Nanodrop ND-1000 spectrophotometer, and adjusted to 200–250 ng/µl in TE buffer (*see* **Subheading 2.1., item 2**). Concentrations are re-measured before dilution of DNA in TE buffer to a final concentration of 20 ng/µl and storage at 4°C.

3.1.2. Sequencing of *PRNP* Open Reading Frame

3.1.2.1. PCR of *PRNP* Open Reading Frame

1. Prepare a premix of MegaMix Royal (a 2x concentrate, *see* **Subheading 2.1., item 3**) containing primers at 0.5 µM sufficient for 25-µl reactions on a 96-well plate. PCR

primers used to amplify the open reading frame are 5′-CTA TGC ACT CAT TCA TTA TGC-3′ (forward) and 5′-GTT TTC CAG TGC CCA TCA GTG-3′ (reverse). Use 12.5 µl MegaMix Royal and make each reaction up to 25 µl using water and primers.

2. Add 1 µl of 20 ng/µl genomic DNA.

3. Thermal cycling is performed on an MJ Research (Watertown, MA) Tetrad 1 PCR machine or similar using the following cycling parameters:

  a. 95 °C for 5 min.

  b. 95 °C for 30 s.

  c. 58 °C for 40 s.

  d. 72 °C for 1 min.

  e. repeat steps b–d an additional 34 times.

4. Assess polymerase chain reaction (PCR) by electrophoresis of 5 µl of product on a 2% ethidium bromide-stained agarose gel with 5 µl of HyperLadder IV (*see* **Subheading 2.1., item 4**) size standard. The gel is viewed using a Gel Doc 1000 transilluminator (Bio-Rad, Hemel Hempstead, UK) and Quantity One 4.5.1 software or similar.

3.1.2.2. PCR Product Cleanup

1. An equal volume of MicroClean (*see* **Subheading 2.1., item 5**) is added to the PCR product and mixed well by pipetting or vortexing.

2. The mixture is left at room temperature for 15 min.

3. The plate is centrifuged at 2,000–4,000*g* for 40 min at room temperature.

4. The supernatant is removed by centrifuging the plate at 40*g* for 30 s in an inverted position on tissue paper by using centrifuge plate holders.

5. Resuspend the cleaned PCR product in 150 µl of water.

3.1.2.3. Sequencing Reactions

1. For each sequencing reaction, prepare a premix of 1 µl of BigDye (*see* **Subheading 2.1., item 6**), 5 µl of BetterBuffer (*see* **Subheading 2.1., item 7**), 0.75 µl of sequencing primer at 5 pmol/µl, approximately 2.5 ng of cleaned PCR product, and water to a final volume of 15 µl. The amount of PCR product is estimated using visual comparison with known amounts of HyperLadder IV (*see* **Subheading 2.1., item 4**) size standard.

2. Sequencing primers are 5′-GAC GTT CTC CTC TTC ATT TT-3′ (forward 1), 5′-CCG AGT AAG CCA AAA ACC AAC-3′ (forward 2), and 5′-CAC CAC CAC TAA AAG GGC TGC-3′ (reverse 1), 5′-TTC ACG ATA GTA ACG GTC C-3′ (reverse 2).

3. Sequencing reactions are thermally cycled on an MJ Research Tetrad 1 PCR machine or similar using the following cycling parameters:

a. 96°C for 1 min.

b. 96°C for 10 s.

c. 50°C for 5 s.

d. 60°C for 4 min.

e. Repeat steps b–d 24 times.

3.1.2.4. Sequencing Product Cleanup

1. To each sequencing reaction, add 3.75 µl of 0.125 M EDTA, pH 8.0 (*see* **Subheading 2.1., item 8**).

2. Add 45 µl of 100% ethanol to each reaction and mix by pipetting.

3. Leave reactions at room temperature for 15 min.

4. Centrifuge the plate at 3,000*g* for 30 min at 4°C.

5. The supernatant is removed by centrifuging the plate at 185*g* for 1 min in an inverted position on tissue paper.

6. Add 50 µl of 70% ethanol in water.

7. Centrifuge the plate at 1,650*g* for 15 min at 4°C.

8. The supernatant is removed by centrifuging the plate at 185*g* for 1 min in an inverted position on tissue paper.

9. Place the plate on the PCR block held at 37°C for 5 min to remove final traces of ethanol.

3.1.2.5. Electrophoresis

1. Add 10 µl of Hi-Di formamide loading solution (*see* **Subheading 2.1., item 9**) and vortex the plate for 30 s.

2. Denature the samples by placing on the PCR block held at 95°C for 2 min and then immediately transfer to ice.

3. Standard run conditions are applied to electrophoresis of sequencing products on an Applied Biosystems 3730xl, using polymer POP7 (*see* **Subheading 2.1., item 10**), 50-cm arrays, and a standard run module with a sample injection time of 15 s.

3.1.2.6. Data Analysis

1. Data analysis is performed using Applied Biosystems Seqscape software version 2.5.

2. Analysis filter settings are adjusted to allow assembly of poor data due to insertions or deletions (maximum mixed bases 95%, maximum Ns 95%, minimum clear length bp of 1, and minimum sample score of 1).

3. Poor data or failed reactions are removed from projects by visual inspection of data.

3.1.3. PCR Size Fractionation to Investigate Insertion or Deletion Variants

1. Prepare a premix of Mega Mix Blue (*see* **Subheading 2.1., item 3**) containing primers at 0.5 μM sufficient for 25-μl reactions on a 96-well plate. PCR primers are 5′-GAC CTG GGC CTC TGC AAG AAG CGC-3′ (forward) and 5′-GGC ACT TCC CAG CAT GTA GCC G-3′ (reverse).

2. Add 1 μl of 20 ng/μl genomic DNA.

3. Thermal cycling is performed on an MJ Research Tetrad 1 PCR machine or similar using the following cycling parameters:

   a. 94°C for 5 min.

   b. 94°C for 30 s.

   c. 65°C for 30 s.

   d. 72°C for 1 min.

   e. Repeat steps b–d 34 times.

   f. 72°C for 5 min.

4. Assess PCR by electrophoresis of 5 µl of product on a 2% ethidium bromide-stained agarose gel with 5 µl of HyperLadder IV (*see* **Subheading 2.1., item 4**) size standard. The gel is viewed using a Bio-Rad Gel Doc 1000 transilluminator and Quantity One 4.5.1 software or similar.

5. 1 OPRD and 6 OPRI controls are run on each gel (**Fig. 3**).

3.1.4. Mutation Confirmation

A second assay is performed to confirm the presence or absence of missense or stop mutations when a predictive genetic test is being carried out. PCR size fractionation, as described above, is sufficient in addition to sequencing when testing for insertion mutants in a predictive setting; however, unexpected or unknown insertion mutations may require cloning to confirm exact base pair composition. Examples of confirmatory assays used to detect the more common *PRNP* missense mutations and cloning methodology are described in **Subheading 3.1.4.1.**

3.1.4.1. Confirmation of P102L

1. Prepare a premix of MegaMix Blue (*see* **Subheading 2.1., item 3**) containing primers at 0.5 µM sufficient for 25-µl reactions on a 96-well plate. PCR primers are 5′-GAC CTG GGC CTC TGC AAG AAG CGC-3′ (forward) and 5′-GGC ACT TCC CAG CAT GTA GCC G-3′ (reverse).

2. Add 1 µl of genomic DNA at 20 ng/µl.

3. Thermal cycling is performed on an MJ Research Tetrad 1 PCR machine or similar using the following cycling parameters:

a. 94°C for 5 min.

b. 94°C for 30 s.

c. 65°C for 30 s.

d. 72°C for 1 min.

e. Repeat steps b–d 34 times.

f. 72°C for 5 min.

4. Prepare restriction endonuclease reaction by adding 10 μl of PCR product, 1 μl of DdeI (*see* **Subheading 2.1., item 11**), 2.5 μl of 10× NEBuffer 3 (*see* **Subheading 2.1., item 11**), and 11.5 μl of H_2O.

5. Incubate reaction at 37°C for 3 h.

6. Electrophorese 10 μl of digested PCR product on a 3% 2:1 MetaSieve agarose (*see* **Subheading 2.1., item 12**) ethidium bromide-stained gel using 5 μl of HyperLadder IV (*see* **Subheading 2.1., item 4**) size standard. The gel is viewed using a Bio-Rad Gel Doc 1000 transilluminator and Quantity One 4.5.1 software.

7. Digested positive and negative controls are run on each gel to visualize mutant DNA (95-, 101-, and 152-bp) and wild-type DNA (101- and 247-bp) fragment patterns.

3.1.4.2. D178N

1. Prepare a premix of MegaMix Blue (*see* **Subheading 2.1., item 3**) containing primers at 0.5 μM sufficient for 25-μl reactions on a 96-well plate. PCR primers are 5′-CTA TGC ACT CAT TCA TTA TGC-3′ (forward) and 5′-GTT TTC CAG TGC CCA TCA GTG-3′ (reverse).

2. Add 1 μl of genomic DNA at 20 ng/μl.

3. Thermal cycling is performed on an MJ Research Tetrad 1 PCR machine or similar using the following cycling parameters:

a. 94°C for 5 min.

b. 94°C for 30 s.

c. 55°C for 40 s.

d. 72°C for 45 s.

e. Repeat steps b–d 34 times.

f. 72°C for 5 min.

4. Prepare restriction endonuclease reaction by adding 10 μl of PCR product, 1 μl of PflFI (*see* **Subheading 2.1., item 13**), 2.5 μl of 10× NEBuffer 4 (*see* **Subheading 2.1., item 13**) and 11.5 μl of H_2O.

5. Incubate reaction at 37°C for 3 h.

6. Electrophorese 10 μl of digested PCR product on a 2% ethidium bromide-stained agarose gel by using 5 μl of HyperLadder IV (*see* **Subheading 2.1., item 4**) size standard. The gel is viewed using a Bio-Rad Gel Doc 1000 transilluminator and Quantity One 4.5.1 software.

7. Digested positive and negative controls are run on each gel to visualize mutant DNA (1015-bp) and wild-type DNA (386- and 629-bp) fragment patterns.

3.1.4.3. E200K

1. Prepare a premix of MegaMix Blue (*see* **Subheading 2.1., item 3**) containing primers at 0.5 μM sufficient for 25-μl reactions on a 96-well plate. PCR primers are 5′-CTA TGC

ACT CAT TCA TTA TGC-3′ (forward) and 5′-GTT TTC CAG TGC CCA TCA GTG-3′ (reverse).

2. Add 1 µl of genomic DNA at 20 ng/µl.

3. Thermal cycling is performed on an MJ Research Tetrad 1 PCR machine or similar using the following cycling parameters:

a. 94°C for 5 min.

b. 94°C for 30 s.

c. 55°C for 40 s.

d. 72°C for 45 s.

e. Repeat steps b–d 34 times.

f. 72°C for 5 min.

4. Prepare restriction endonuclease reaction by adding 10 µl of PCR product, 1 µl of BsaI (*see* **Subheading 2.1., item 14**), 2.5 µl of 10× NEBuffer 3 (*see* **Subheading 2.1., item 14**), and 11.5 µl of H_2O.

5. Incubate reaction at 50°C for 3 h.

6. Electrophorese 10 µl of digested PCR product on a 2% ethidium bromide-stained agarose gel by using 5 µl of HyperLadder IV (*see* **Subheading 2.1., item 4**) size standard and view using a Bio-Rad Gel Doc 1000 transilluminator and Quantity One 4.5.1 software.

7. Digested positive and negative controls are run on each gel to visualize mutant DNA (1015-bp) and wild-type DNA (318- and 697-bp) fragment patterns.

3.1.5. Characterization of Insertion Mutations

3.1.5.1. Generation of Amplicon to Be Cloned

1. Prepare a premix of MegaMix Blue (*see* **Subheading 2.1., item 3**) containing primers at 0.5 µM sufficient for 25-µl reactions on a 96-well plate. PCR primers are 5′-GAC CTG GGC CTC TGC AAG AAG CGC-3′ (forward) and 5′-GGC ACT TCC CAG CAT GTA GCC G-3′ (reverse). (Note that MegaMix Blue contains an enzyme that has 3-prime terminal adenosine triphosphate transferase activity that ensures that the amplicon has "A" overhangs to anneal to the "T" overhangs of the cloning vector).

2. Add 1 µl of genomic DNA at 20 ng/µl.

3. Thermal cycling is performed on an MJ Research Tetrad 1 PCR machine or similar using the following cycling parameters:

   a. 94°C for 5 min.

   b. 94°C for 30 s.

   c. 65°C for 30 s.

   d. 72°C for 1 min.

   e. Repeat steps b–d 34 times.

   f. 72°C for 10 min.

4. Electrophorese 5 µl of PCR product on a 2% ethidium bromide-stained agarose gel by using 5 µl of HyperLadder IV (*see* Subheading 2.1., item 15) size standard and view using a Bio-Rad Gel Doc 1000 transilluminator and Quantity One 4.5.1 software.

5. To preserve the "A" overhangs, before cloning, carry out as little manipulation as possible and use fresh product.

3.1.5.2. Ligation and Cloning

1. Perform TOPO TA cloning as described in the online user manual version O, 10 April 2006 (25-0276). Updates of this protocol are available at www.invitrogen.com. Use Invitrogen cat. no. K4575-01 (*see* **Subheading 2.1., item 15**) (TOP10, Chemically Competent *E. coli*, 20 reactions).

3.1.5.3. Analysis and Sequencing of Recombinant Clones

1. There is no need to miniprep possible positive clones. Pick white and light blue clones (color enhancement can be obtained by leaving the plate at 4°C overnight if this is preferred) and inoculate wells of a prewarmed 96-well tissue culture or storage plate containing 150–200 µl of LB broth (*see* **Subheading 2.1., item 16**) containing the appropriate antibiotic.

2. Incubate at 37°C for about 5–6 h or until the wells are opaque.

3. Transfer 50-µl aliquots to a 96-well PCR plate.

4. Seal the plate and place on a PCR machine for 10 min at 99°C to lyse the bacteria. Aliquots (1 µl) of these crude DNA preparations can then be used to produce amplicons by using the same methods used to produce the original amplicon by simple transfer to a fresh 96-well PCR plate containing the appropriate PCR mix.

5. Electrophorese 5 µl of PCR product on a 2% ethidium bromide-stained agarose gel by using 5 µl of HyperLadder IV (*see* **Subheading 2.1., item 4**) size standard. The gel is viewed using a Bio-Rad Gel Doc 1000 transilluminator and Quantity One 4.5.1 software.

6. Amplicons from positive wells can be purified and sequenced according to the automated sequencing protocol for PCR products (*see* **Subheadings 3.1.2.2.–3.1.2.6.**).

7. Amplicons will contain Taq polymerase artefacts preserved in the cloning process. Therefore, at least three clones should be sequenced to obtain a consensus sequence, preferably on both strands.

3.1.6 Alternative Confirmation of Missense or Stop Mutations using Real-Time PCR

3.1.6.1 P102L

1. For each reaction, prepare a premix of 10 μl of GTXpress Master Mix (*see* **Subheading 2.1., item 17**), 2 μl each of forward primer, reverse primer, wild-type probe and mutant probe and 1 μl of water (Primer stocks at 9 μM, probe stocks are at 2 μM). Primer and probes are 5'-GGA GGT GGC ACC CAC AGT C-3' (forward primer), 5'-GCC ATG TGC TTC ATG TTG GTT-3' (reverse primer), 5'-FAM-CTT ACT CGG CTT GTT C-3' (wild-type probe) and 5'-VIC-CTT ACT CAG CTT GTT CC-3' (mutant probe) (*see* **Subheading 2.1., item 18**).
2. Add 1 μl of genomic DNA at 20 ng/μl. Positive, negative and no-template controls are also run on each plate.
3. Thermal cycling and data capture are performed on a QuantStudio 12K Flex machine (Life Technologies) using a fast 96-well block, choosing genotyping mode and a fast instrument run.
4. On completion of the run, inspecting the allele discrimination plot will show the unknown sample clustering with either the positive controls or the negative controls.

3.1.6.2 D178N

1. For each reaction, prepare a premix of 10 µl of GTXpress Master Mix (*see* **Subheading 2.1., item 17**), 2 µl each of forward primer, reverse primer, wild-type probe and mutant probe and 1 µl of water. Primer and probes are 5'-CAG GCC CAT GGA TGA GTA CA-3' (forward primer), 5'-CGT GTG CTG CTT GAT TGT GA-3' (reverse primer), 5'-FAM-TTG ACG CAG TCG TGC A-3' (wild-type probe) and 5'-VIC-TTG ACG CAG TTG TGC A-3' (mutant probe) (*see* **Subheading 2.1., item 18**).
2. Add 1 µl of genomic DNA at 20 ng/µl. Positive, negative and no-template controls are also run on each plate.
3. Thermal cycling and data capture are performed on a QuantStudio 12K Flex machine (Life Technologies) using a fast 96-well block, choosing genotyping mode and a fast instrument run.
4. On completion of the run, inspecting the allele discrimination plot will show the unknown sample clustering with either the positive controls or the negative controls.

3.1.6.3 E200K

1. For each reaction, prepare a premix of 10 µl of GTXpress Master Mix (*see* **Subheading 2.1., item 17**), 2 µl each of forward primer, reverse primer, wild-type probe and mutant probe and 1 µl of water. Primer and probes are 5'-CGG TCA CCA CAA CCA CCA A-3' (forward primer), 5'-CAC GCG CTC CAT CAT CTT AA-3' (reverse primer), 5'-FAM-AAC TTC ACC GAG ACC GA-3' (wild-type probe) and 5'-VIC-AGA ACT TCA CCA AGA CC-3' (mutant probe) (*see* **Subheading 2.1., item 18**).
2. Add 1 µl of genomic DNA at 20 ng/µl. Positive, negative and no-template controls are also run on each plate.

3. Thermal cycling and data capture are performed on a QuantStudio 12K Flex machine (Life Technologies) using a fast 96-well block, choosing genotyping mode and a fast instrument run.

4. On completion of the run, inspecting the allele discrimination plot will show the unknown sample clustering with either the positive controls or the negative controls.

3.2. *Immunoblotting*

3.2.1. Biosafety

1. All procedures are performed within a class 1 microbiological safety cabinet situated within an ACDP level III containment laboratory with strict adherence to local rules of safe working practice. Informed consent for the analysis of samples must be in place before investigation.

2. No unsealed biological material (tissue or derivative sample thereof) is manipulated outside of the class 1 microbiological safety cabinet. Disposable gloves, safety gown, and safety glasses are worn at all times.

3. 1.5-ml screw-top microfuge tubes containing a rubber O-ring are used.

4. Guidelines for decontamination of human prions are available ***(75)***. All disposable plasticware (e.g., tubes, tips, and so on) and solutions containing biological material are decontaminated in 50% (v/v) sodium hypochlorite solution (containing >20,000 ppm available chlorine prepared in water) for at least 1 h before disposal of the liquid phase down designated laboratory sinks within the containment laboratory. Sharps (needles and scalpels) are disposed of immediately after use into a sharps bin and autoclaved at 136°C for 20 min before incineration.

5. Decontaminated plasticware is transferred to a sharps bin and autoclaved at 136°C for 20 min before incineration.

3.2.2. Preparation of Tissue Homogenate

1. Tissue specimens, stored frozen in sealed pots within the ACDP level III containment laboratory, are transferred into a class 1 microbiological safety cabinet and partially thawed and placed on a petri dish.

2. A suitable quantity of tissue is excised using a scalpel and sealed in a disposable plastic pot and weighed. The tissue is then prepared as a 10% (w/v) homogenate in Dulbecco's sterile PBS lacking Ca^{2+} and Mg^{2+} ions (*see* **Subheading 2.2., item 1**). The amount of PBS to add in microliters is equal to 9 times wet weight of tissue in milligrams. This calculation will produce a homogenate very close to a true 10% (w/v) ratio without the necessity of having to accurately measure the total volume of tissue in PBS before the homogenization process.

3. Homogenization of brain tissue is achieved by serial passage of tissue through syringe needles of decreasing diameter (needle gauges 19, 21, 23, and 25).

4. Homogenization of peripheral tissue is achieved through the use of glass Duall tissue grinders (*see* **Subheading 2.2., item 2**).

5. The homogenate is stored as aliquots in 1.5 ml screw-top microfuge tubes at –80°C.

3.2.3. Proteinase K Digestion and Electrophoresis

1. 10% brain homogenate is thawed, thoroughly vortexed, and then centrifuged at 100*g* (800 rpm) for 1 min in a microfuge (*see* **Note 1**).

2. 20-μl aliquots of the resultant supernatant are adjusted to a final concentration of 50 μg/ml proteinase (*see* **Subheading 2.2., item 3**) by addition of 1.05 μl of a 1 mg/ml proteinase K stock solution (*see* **Note 2**).

3. Samples are incubated at 37°C for 1 h, followed by centrifugation at 16,100*g* (13,200 rpm) for 1 min in a microfuge.

4. The digestion is terminated by resuspension of the sample with an equal volume (21 μl) of 2× working SDS sample buffer (*see* **Subheading 2.2., item 4**) and *immediate* transfer to a 100°C heating block for 10 min.

5. Samples for analysis in the absence of proteinase K treatment are treated directly with an equal volume of 2× working SDS sample buffer (*see* **Subheading 2.2., item 4**) and heated similarly.

6. All samples are centrifuged at 16,100*g* (13,200 rpm) for 1 min in a microfuge, thoroughly vortexed, and then recentrifuged 16,100*g* for 1 min before electrophoresis of the supernatant.

7. 10 μl of the supernatant is loaded on an Novex® 16% Tris-glycine polyacrylamide mini gel (*see* **Subheading 2.2., item 5**) (*see* **Note 3**). The remainder of the sample can be stored at –80°C. Then, 10 μl of 1× working SDS sample buffer (prepared by mixing 2× working SDS sample buffer with an equal volume of water) should be added in any blank lane. Ten microliters of Seeblue prestained molecular mass markers (*see* **Subheading 2.2., item 6**) is used to calibrate the gel.

8. Gels are run at a constant voltage of 200 V for 80 min in SDS-PAGE running buffer (*see* **Subheading 2.2., item 7**) (*see* **Note 3**).

9. Gels are electroblotted (one gel per XCell II™ blot module (Novex®)) on to polyvinylidene difluoride membrane (*see* **Subheading 2.2., item 8**) in electroblotting buffer (*see* **Subheading 2.2., item 9**) at a constant voltage of 35 V for 2 h or 15 V overnight. Immobilon P membrane is soaked for 2 min in 100% methanol and then rinsed in electroblotting buffer immediately before use.

3.2.4. High-Sensitivity Chemiluminescence (ECL)

1. Blots are blocked with 5% (w/v) nonfat milk powder in PBST (*see* **Subheading 2.2., item 10**) for 1 h followed by brief rinsing with PBST.
2. Blots are incubated with anti-PrP monoclonal antibody 3F4 (*see* **Subheading 2.2., item 11**) at a final concentration of 0.2 μg/ml in PBST containing 0.1% (w/v) sodium azide for either 90 min or overnight.
3. Blots are washed for a minimum of 30 min and up to 60 min with at least six changes of PBST.
4. Blots are incubated for 1 h with a 1:10,000 dilution of goat anti-mouse IgG-phosphatase conjugate (*see* **Subheading 2.2., item 12**) in PBST.
5. Blots are washed for a minimum of 30 min and up to 60 min with at least six changes of PBST.
6. Blots are washed 2 × 5 min with 20 mM Tris, pH 9.8, containing 1 mM $MgCl_2$.
7. Blots are developed with chemiluminescent substrate CDP-Star (*see* **Subheading 2.2., item 13**) and visualized on Biomax MR film (*see* **Subheading 2.2., item 14**) (*see* **Note 4**).

3.2.5. Standard Enhanced Chemifluorescence (ECF)

1. Blots are blocked with 5% (w/v) nonfat milk powder in PBST (*see* **Subheading 2.2., item 10**) for 1 h followed by brief rinsing with PBST.

2. Blots are incubated with anti-PrP monoclonal antibody 3F4 (*see* **Subheading 2.2., item 11**) at a final concentration of 0.2 μg/ml in PBST containing 0.1% (w/v) sodium azide for either 90 min or overnight.

3. Blots are washed for a minimum of 30 min and up to 60 min with at least six changes of PBST.

4. Blots are incubated for 1 h with a 1:5,000 dilution of goat anti-mouse IgG-phosphatase conjugate (*see* **Subheading 2.2., item 12**) in PBST.

5. Blots are washed for a minimum of 30 min and up to 60 min with at least six changes of PBST.

6. Blots are washed 2 × 5 min with 20 mM Tris, pH 9.8, containing 1 mM $MgCl_2$.

7. Blots are developed with chemifluorescent substrate AttoPhos (*see* **Subheading 2.2., item 15**) and visualized on a Storm 840 PhosphorImager (GE Healthcare, Little Chalfont, Buckinghamshire, UK). PrP glycoforms are quantified with ImageQuaNT software (GE Healthcare) (*see* **Note 4**).

3.2.6. Sodium Phosphotungstic Acid Precipitation

Methods are adapted from the original procedure of Safar et al. ***(76)*** as described by Wadsworth et al. (***35***).

1. 10% (w/v) homogenates from human brain or peripheral tissues prepared in Dulbecco's PBS lacking Ca^{2+} and Mg^{2+} ions (*see* **Subheading 2.2., item 1**) are centrifuged at 100*g* (800 rpm) for 1 min in a microfuge.

2. 500 µl of the resultant supernatant is mixed with an equal volume of 4% (w/v) sodium lauroylsarcosine (*see* **Subheading 2.2., item 16**) prepared in Dulbecco's PBS lacking Ca^{2+} and Mg^{2+} ions (*see* **Subheading 2.2., item 1**) and incubated for 10 min at 37°C with constant agitation.

3. Samples are adjusted to final concentrations of 50 U/ml Benzonase (*see* **Subheading 2.2., item 17**) (add 2 µl of 25 U/µl Benzon nuclease, purity 1) and 1 mM $MgCl_2$ (add 0.5 µl of 2 M $MgCl_2$ prepared in water) and incubated for 30 min at 37°C with constant agitation.

4. Samples are adjusted with 81.3 µl of a sodium phosphotungstic acid stock solution (*see* **Subheading 2.2., item 18**) to give a final concentration in the sample of 0.3% (w/v) sodium phosphotungstic acid. This stock solution is pre-warmed to 37°C before use, and both the sample and the stock solution should be at 37°C upon mixing to avoid formation of insoluble magnesium salts.

5. Samples are incubated at 37°C for 30 min with constant agitation before centrifugation at 16,100*g* (13,200 rpm) for 30 min in a microfuge. The microfuge rotor can be prewarmed to 37°C before use, because this helps to avoid salt precipitation during centrifugation.

6. After careful isolation of the supernatant, the sample is recentrifuged at 16,100*g* (13,200 rpm) for 2 min, and the residual supernatant is discarded. New tops are placed on the microfuge tubes.

7. Pellets are resuspended to a 20-μl final volume with Dulbecco's PBS lacking Ca^{2+} and Mg^{2+} ions containing 0.1% (w/v) sodium lauroylsarcosine and proteinase K digested and processed for immunoblotting as described in **Subheading, 3.2.3, steps 2-9** (*see* **Note 5**).

3.3. Immunohistochemistry

3.3.1. Procurement

3.3.1.1. Biosafety

For samples suspected to contain infectious prions, all procedures are performed within a class 1 microbiological safety cabinet situated within an ACDP level III containment laboratory with strict adherence to local rules of safe working practice. Informed consent for the analysis of samples must be in place before investigation. Samples are kept in a category III laboratory before decontamination with formic acid. Guidelines for decontamination of human prions are available ***(75)***. Liquids that have been in contact with infected samples are decontaminated by mixing with an equal volume of 2 M sodium hydroxide for at least 1 h. For certain reagents, specialist disposal is preferred due to chemical incompatibilities ***(75)***.

3.3.1.2. Whole Brain, Brain Hemispheres, or Whole Internal Organs

1. Large specimens of tissue (whole brain, brain hemispheres, whole internal organs) are suspended in 10% buffered formal-saline (*see* **Subheading 2.3.1., item 1**). The volume added should be approximately 5 times the volume of tissue.
2. If there is excess blood within the sample, the 10% buffered formal-saline should be exchanged until it remains clear.

3. Tissue is left for up to 3 weeks to ensure adequate fixation and hardening.

4. After fixation, samples of tissue are excised with dimensions suitable for histology cassettes (*see* **Subheading 2.3.1., item 2**).

3.3.1.3. Small Specimens of Brain or Peripheral Tissues

1. Smaller pieces of brain (frontal cortex, temporal cortex, parietal cortex, occipital cortex, cerebellum) or samples of other peripheral tissues (tonsil, spleen, lymph nodes, appendix), with dimensions no larger than approximately 3 cm × 3 cm × 1 cm, are commonly provided for investigation.

2. Tissue samples are immersed in approximately 5 volumes of 10% buffered formal-saline (*see* **Subheading 2.3.1., item 1**).

3. If there is excess blood within the sample, the 10% buffered formal-saline should be exchanged until it remains clear.

4. Fixation of the samples is achieved after 2 days.

5. After fixation, samples of tissue are excised with dimensions suitable for histology cassettes (*see* **Subheading 2.3.1., item 2**).

3.3.2. Prion Deactivation with Formic Acid

1. All brain tissue must be of a size suitable for processing. Generally, this is considered as the size and thickness of the histology cassettes (*see* **Subheading 2.3.2., item 1**). Care must be taken not to overfill the cassettes, because this will result in poor processing and distortion.

2. After being encased in labeled cassettes, the samples are immersed in 98% formic acid (*see* **Subheading 2.3.2., item 2**) for 1 h.

3. Formic acid is decanted into a waste pot half filled with 2 M sodium hydroxide (*see* **Subheading 2.3.2., item 3**).

4. Specimens are treated with approximately5 volumes of 10% buffered formal-saline (*see* **Subheading 2.3.2., item 4**) for 1 h.

5. The 10% buffered formal-saline (*see* **Subheading 2.3.2., item 4**) is exchanged at least once to ensure any excess of formic acid has been removed before tissue processing.

6. Samples are removed from the ACDP level III containment laboratory.

7. Samples are placed on a tissue processor in an ACDP level II containment laboratory.

3.3.3. Tissue Processing

10% buffered formal saline (*see* **Subheading 2.3.3., item 1**) is an aqueous fixative; therefore, the samples are treated through a series of processing stages before wax embedding. Each stage needs to be of sufficient length to ensure impregnation. The stages are as follows:

1. Dehydration: The samples are taken through a series of industrial methylated spirits (IMS) (*see* **Subheading 2.3.3., item 2**) (70, 90, 100%) to remove water (**Table 1**).

2. Clearing: The alcohol is replaced by xylene (*see* **Subheading 2.3.3., item 3**), a fluid miscible with IMS and paraffin wax (*see* **Subheading 2.3.3., item 4**) (**Table 1**).

3. Impregnation: the xylene is replaced with molten paraffin wax (*see* **Subheading 2.3.3., item 4**) (**Table 1**).

4. Embedding: The samples are embedded in the desired orientation in molten paraffin wax (*see* **Subheading 2.3.3., item 4**). Once the wax has hardened, the samples are ready for sectioning.

3.3.4. Tissue Sectioning

1. The microtome (*see* **Subheading 2.3.4., item 1**) is set at 8 μm for tissue sectioning, although this measure can be varied.

2. The sample block, now in wax, is mounted on to the microtome chuck and serial sections of the sample are taken.

3. Sections are floated out on a water bath set at 40°C.

4. The sections are mounted on SuperFrost microscope slides (*see* **Subheading 2.3.4., item 2**) and left to air dry at 37°C for approximately 2 h.

5. Slides are dried at 60°C for a minimum of 2 h, after which they are ready to be stained.

6. Tonsil sections require cutting just before staining. Immunoreactivity is markedly reduced if sections are exposed to air for long periods of time.

3.3.5. Tissue Staining

3.3.5.1. Staining with Hematoxylin and Eosin (H&E)

1. Rehydrate the sections by removing paraffin in three changes of xylene (*see* **Subheading 2.3.5., item 1**), followed by sequential washing for 1–2 min with graded alcohol (*see* **Subheading 2.3.5., item 2**) (100% × 2, 90%, and 70%) and final washing in running tap water.

2. Place the slides in filtered Harris hematoxylin solution (*see* **Subheading 2.3.5., item 3**) for 5 min.

3. Wash briefly in running tap water and differentiate in 1% acid alcohol (*see* **Subheading 2.3.5., item 4**) for 30 s.

4. Wash well in running tap water and allow the color to develop. Check microscopically. Nuclei look dark blue, whereas background shows a weak residual hematoxylin coloration.

5. Wash briefly in running water and stain with Eosin Y solution (*see* **Subheading 2.3.5., item 5**) for 2–3 min.

6. Wash sections sequentially for ~1–2 min with water, 70% ethanol, 90% ethanol, 100% ethanol, and xylene.

7. Mount sections in a xylene-based mounting medium, Pertex (*see* **Subheading 2.3.5., item 6**) (*see* **Note 6**).

3.3.5.2. Staining for Glial Fibrillary Acidic Protein (GFAP)

1. The sections to be stained are placed in plastic racks and paraffin removed, as described in **Subheading 3.3.5.1.**

2. The slides are placed on the Benchmark XT Staining Machine (*see* **Subheading 2.3.5., item 7**) (Ventana Medical Systems) with a 4-min pre-treatment with Protease 1 (*see* **Subheading 2.3.5., item 8**).

3. The slides are incubated with a GFAP antibody (*see* **Subheading 2.3.5., item 9**) diluted 1:1000 in antibody diluent (*see* **Subheading 2.3.5., item 9**).

4. The slides are stained using the staining kit IViewDAB (*see* **Subheading 2.3.5., item 10**) and counterstained using hematoxylin (*see* **Subheading 2.3.5., item 11**) and a bluing reagent (*see* **Subheading 2.3.5., item 12**).

5. Once the run is finished, the slides are washed in hot soapy water (diluted washing up liquid) and dehydrated through alcohol and xylene.

6. Mount sections in a xylene-based mounting medium, Pertex (*see* **Subheading 2.3.5., item 6**) (*see* **Note 7**).

3.3.5.3. Staining for PrP

1. The sections to be stained are placed in plastic racks and paraffin removed as described in **Subheading 3.3.5.1.**

2. The pre-treatment for detection of abnormal PrP deposition is dependent upon the tissue and the length of fixation. For human brain samples that have been fixed for up to ~2 weeks, the microwave heat retrieval method is preferred. If the brain samples are fixed for longer periods, the pressure cooker method is used. If tonsil or other secondary lymphoid tissue is being examined, the autoclaving heat retrieval method is used.

3.*3.5.3.1. Microwave Method*

3. After removal of paraffin (*see* **Subheading 3.3.5.1.**) the slides are placed in 1 liter of Tris-EDTA-citrate buffer (*see* **Subheading 2.3.5., item 13**), and then they were placed in a microwave for 25 min at 800-W power.

4. The slides are washed in running cold tap water for 3 min.

5. The samples are covered with 98% formic acid (*see* **Subheading 2.3.5., item 14**), incubated for 5 min, and then washed in running cold tap water for 5 min to remove excess formic acid.

3.3.5.3.2. Pressure Cooker Method

6. After removal of paraffin (*see* **Subheading 3.3.5.1.**), the slides are placed in a pressure cooker containing 1.5 liters of boiling Tris-EDTA-citrate buffer (*see* **Subheading 2.3.5., item 13**) for 5 min at high pressure and 5 min at low pressure.
7. Place the slides under running cold water for 5 min and treat with 98% formic acid (*see* **Subheading 2.3.5., item 14**) for a further 5 min. Wash the slides in running water for 5 min to remove excess formic acid.

3.3.5.3.3. Autoclaving Heat Retrieval

8. After removal of paraffin (*see* **Subheading 3.3.5.1.**), the slides are placed in an autoclave resistant tub containing 1 liter of citrate buffer (*see* **Subheading 2.3.5., item 15**).
9. The tub is covered with aluminium foil and run in an autoclave at 121°C for 20 min. Allow autoclave to return to low pressure before removing the tub. Place slides under running cold tap water.
10. Treat with 98% formic acid (*see* **Subheading 2.3.5., item 14**) for a further 5 min. Wash the slides in running water for 5 min to remove excess formic acid.

3.3.5.3.4. Automated Staining

11. The monoclonal anti-PrP antibody ICSM35 (*see* **Subheading 2.3.5., item 16**) (1 mg/ml stock) is used at a 1:1,000 dilution in antibody diluent (*see* **Subheading 2.3.5., item 9**).

12. Automated staining is carried out on the Benchmark Staining Machine (*see* **Subheading 2.3.5., item 7**) from Ventana Medical Systems.

13. The slides are subjected to further pre-treatment with Protease 3 (*see* **Subheading 2.3.5., item 17**) for 4 min, and then 10 min with Superblock (*see* **Subheading 2.3.5., item 18**), a blocking agent. The slides are stained using the staining kit IViewDAB (*see* **Subheading 2.3.5., item 10**) and counterstained using hematoxylin (*see* **Subheading 2.3.5., item 11**) and a bluing reagent (*see* **Subheading 2.3.5., item 12**).

14. Once the staining process is complete, the slides are washed in hot soapy water (diluted washing-up liquid) and dehydrated through alcohol and xylene. They are mounted in a xylene-based mountant as described in **Subheading 3.3.5.1.** (*see* **Note 8**).

3.3.5.3.5. Manual Staining

15. Sections are treated to remove paraffin as far as 100% alcohol (*see* **Subheading 3.3.5.1.**).

16. Block endogenous peroxidase activity on the sections by treatment with 2.5% (v/v) hydrogen peroxide in methanol (*see* **Subheading 2.3.5., item 19**) for 30 min.

17. Wash sections in running tap water for 5 min and then in purified water for 5 min and transfer to an appropriate container for autoclaving. Autoclave at 121°C for 20 min in Tris-buffered saline (TBS), pH 7.6 (*see* **Subheading 2.3.5., item 20**).

18. Cool slides in running tap water. Treat the slides in 98% formic acid (*see* **Subheading 2.3.5., item 14**) for 5 min, wash in running tap water for 5–10 min.

19. Treat sections with 4 M guanidine thiocyanate (*see* **Subheading 2.3.5., item 21**) at 4°C for 2 h and then wash in tap water and transfer to TBS (*see* **Subheading 2.3.5., item 20**).

20. Block nonspecific immunoglobulin staining with normal rabbit serum (*see* **Subheading 2.3.5., item 22**) diluted 1:10 in TBS for 30 min. Do not wash off.

21. Apply primary antibody ICSM35 (*see* **Subheading 2.3.5., item 16**) (1 mg/ml stock) at 1:1500 dilution in TBS containing 1:100 normal rabbit serum (*see* **Subheading 2.3.5., item 22**), overnight at 4°C.

22. Wash in several changes of TBS.

23. Incubate in biotinylated rabbit anti-mouse immunoglobulins (*see* **Subheading 2.3.5., item 23**) 1:200 in TBS for 45 min.

24. Wash in several changes of TBS.

25. Incubate in ABC complex (*see* **Subheading 2.3.5., item 24**) for 45 min.

26. Wash in several changes of TBS.

27. Develop in 3,3 diaminobenzidine tetrachloride (*see* **Subheading 2.3.5., item 25** (25 mg/100 ml of TBS) plus 30 µl of hydrogen peroxide (*see* **Subheading 2.3.5., item 19**) (added just before use) for 5–15 min. Check microscopically. Once chromogen has developed to satisfaction, wash slides in running tap water for 10 min.

28. Counterstain in Harris hematoxylin (*see* **Subheading 2.3.5., item 3**) for 3 min.

29. Differentiate in 1% acid alcohol (*see* **Subheading 2.3.5., item 4**) for 5 s.

30. Allow blue coloration to develop in tap water, 5 min.

31. Dehydrate, clear, and mount as described in **Subheading 3.3.5.1.** (*see* **Note 8**).

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4. Notes

1. Whole brain homogenate can be analyzed by identical procedures; however problems of high sample viscosity due to nucleic acid aggregation are often encountered. For processing of 20 μl of whole brain homogenate, preincubation with 0.5 μl of Benzonase for 10 min at 20°C is recommended before further sample analysis by using appropriately adjusted volumes of subsequent reagents.

2. PrP^{Sc} is covalently indistinguishable from PrP^{C}, but it can by differentiated from PrP^{C} by its partial resistance to proteolysis and its marked insolubility in detergents ***(1, 77)***. Under conditions in which PrP^{C} is completely degraded by the nonspecific protease, proteinase K, PrP^{Sc} in sporadic and acquired forms of human prion disease exists in an aggregated form with the C-terminal two thirds of the protein showing marked resistance to proteolysis, leading to the generation of amino terminally truncated fragments of di-, mono- and nonglycosylated PrP ***(1, 77)*** (**Fig. 4**).

3. The procedures described here have been optimized for use with Novex® 16% acrylamide precast Tris-glycine gels. Variation in the resolution of the system may occur if other gel systems are used or if reagent compositions are varied from those listed here. Optimal resolution of PrP^{Sc} fragment size is achieved after electrophoresis for 80 min at 200 V. For improved separation of PrP glycoforms for densitometry analysis, electrophoresis is performed for 90 min at 200 V.

4. To date, we have identified four major types of human PrP^{Sc} associated with sporadic and acquired human prion diseases that can be differentiated by their fragment size on immunoblots after limited proteinase K digestion of brain homogenates ***(7, 9, 78, 79)*** **(Fig. 4)**. These types can be further classified by the ratio of the three PrP bands seen after protease digestion, corresponding to amino-terminally truncated cleavage products generated from di-, mono-, or nonglycosylated PrP^{Sc} (**Figs. 4, 5**). PrP^{Sc} types 1–3 are seen in classical (sporadic or iatrogenic) CJD brain, whereas type 4 PrP^{Sc} is uniquely seen in vCJD brain ***(7, 9, 78, 80)***. An earlier classification of PrP^{Sc} types seen in classical CJD described only two banding patterns ***(81)*** with PrP^{Sc} types 1 and 2 that we describe corresponding with the type 1 pattern of Gambetti and colleagues, and our type 3

fragment size corresponding to their type 2 pattern ***(8, 82)***. Although type 4 PrP^{Sc} is readily distinguished from the PrP^{Sc} types seen in classical CJD by a predominance of the diglycosylated PrP glycoform, type 4 PrP^{Sc} also has a distinct proteolytic fragment size ***(9)*** (**Fig. 4**), although this is not recognized by the alternative classification, which designates type 4 PrP^{Sc} as type 2b ***(82)***. Although proteinase K-resistant PrP fragments of ~21–30 kDa seen in inherited prion disease caused by *PRNP* P102L, D178N, and E200K mutations have molecular masses similar in size to those seen in classical CJD ***(10, 83-85)***, the glycoform ratio is distinct from PrP^{Sc} fragments seen in both classical CJD ***(10, 83-85)*** and vCJD ***(10)*** (**Fig. 5**). Individuals with these mutations also propagate PrP^{Sc} with distinct fragment sizes ***(10, 83, 84)***. The fragment sizes and glycoform ratios of PrP^{Sc} seen in 2-, 4-, and 6-OPRI cases are indistinguishable from those of PrP^{Sc} seen in classical CJD ***(10)***. Importantly detection of PrP^{Sc} in the molecular mass range of ~21–30 kDa is by no means a consistent feature in inherited prion disease; and some cases, in particular those in which amyloid plaques are a prominent feature, show smaller protease resistant PrP fragments of ~7–15 kDa derived from the central portion of PrP ***(10, 21, 83, 84, 86-88)*** (**Fig. 4**).

5. Sodium phosphotungstic acid precipitation facilitates highly efficient recovery and detection of PrP^{Sc} from human tissue homogenate, even when present at levels 10^4–10^5-fold lower than found in brain ***(35, 89)***. This procedure is now the preferred method for diagnostic analysis of tonsil in cases of suspected vCJD, and it should detect PrP^{Sc} in tonsil if levels reach 0.1% or above the maximum levels seen in necropsy vCJD tonsil ***(35, 90)***. A distinctive PrP^{Sc} type, designated type 4t, is seen in both ante-mortem and post-mortem tonsil from patients with vCJD ***(35, 68)*** (*see* **Figs. 5 and 7**), including

secondary vCJD infection resulting from blood transfusion ***(38)***. Type 4t PrP^{Sc} in tonsil differs in the proportions of the PrP glycoforms from type 4 PrP^{Sc} seen in vCJD brain ***(35, 68)*** (*see* **Figs. 5 and 7**), implying the superimposition of tissue and strain specific effects on PrP glycosylation ***(32, 68)***.

6. On H&E-stained sections nuclei are stained deep blue, and the cytoplasm is stained pink. The cortex and subcortical white matter can be readily distinguished. In the cortex of a patient with prion disease, there may be variable degrees of spongiosis, accompanied astroglial proliferation (**Fig. 6**). Neuronal loss also may be evident. Although synaptic PrP deposition is generally not recognizable on H&E sections, amyloid PrP plaques as seen in GSS and vCJD may be a prominent feature (**Fig. 6**). In the cerebellum, spongiosis is generally less evident; however, PrP plaques may be observed particularly in GSS.

7. Reactive astrocytes are readily visualized by GFAP immunohistochemistry. They are characterized by prominent processes (**Fig. 6**). In the white matter, there may be a diffuse fibrillary gliosis.

8. Abnormal PrP deposition can present with a multitude of intensities, shapes, and distributions. The *synaptic* pattern is characterized by a fine, dispersed distribution, and it is the predominant pattern of abnormal PrP staining seen in sporadic CJD (**Fig. 6**). In contrast, PrP amyloid plaques are a predominant feature in GSS, kuru, and vCJD. The histopathologic features of vCJD are remarkably consistent and distinguish it from other human prion diseases with large numbers of PrP-positive amyloid plaques that differ in morphology from the plaques seen in kuru and GSS in that the surrounding tissue takes on a microvacuolated appearance, giving the plaques a florid appearance (**Fig. 6**).

Abnormal PrP immunoreactivity in vCJD tonsil ***(38, 68, 90)*** and appendix ***(34, 91)*** is confined to lymphatic follicles with deposition mainly in dendritic cells (**Fig. 7**).

Figure legends

Fig. 1. Pathogenic mutations and polymorphisms in human prion protein. The pathogenic mutations associated with human prion disease are shown above the human PrP coding sequence. These consist of four to twelve OPRI within the octapeptide repeat region between codons 51 and 91, a two OPRD, and various point mutations causing missense or stop codon substitutions. Some of these changes have been observed in individual patients only and should be considered as possible pathogenic mutations that require confirmation. Point mutations are designated by the wild-type amino acid preceding the codon number, followed by the mutant residue, using single-letter amino acid nomenclature (X denotes stop). Polymorphic variants are shown below the PrP coding sequence (synonymous changes, *green*; nonsynonymous changes, *blue*). Codon 129 and 219 polymorphisms have profound susceptibility, disease-modifying effects, or both. Deletion of one octapeptide repeat is not associated with prion disease in humans.

Fig. 2. *PRNP* codon 129 polymorphism. Electropherogram traces that illustrate all *PRNP* codon 129 genotypes in the reverse DNA strand orientation.

Fig. 3. Analysis of *PRNP* OPRI mutations. Image from agarose gel electrophoresis of *PRNP* amplicons illustrating the presence of an insertional mutation. Lane 1, HyperLadder IV; lane 2, 1-OPRD control; lane 3, 6 OPRI control; lane 4–8, patient

samples: lane 6 demonstrates amplification of a heterozygous insertional mutation of 144 base pairs (6-OPRI mutation positive), lanes 4, 5, 7, and 8 are wild-type alleles only; lane 9, no-template control.

Fig. 4. Immunoblot analysis of human PrP. **(A)** Immunoblot analysis of normal human brain and vCJD brain homogenate before and after treatment with proteinase K (PK). PrP^{C} in both normal and vCJD brain is completely degraded by PK, whereas PrP^{Sc} present in vCJD brain shows resistance to proteolytic degradation leading to the generation of amino terminally truncated fragments of di-, mono-, and nonglycosylated PrP. **(B)** Immunoblot of PK digested brain homogenate with monoclonal antibody 3F4 showing PrP^{Sc} types 1–4 in human brain. Types 1–3 PrP^{Sc} are seen in the brain of classical forms of CJD (either sporadic or iatrogenic CJD), whereas type 4 PrP^{Sc} is uniquely seen in vCJD brain. Classification according to Hill et al. ***(9)***. **(C)** Immunoblots of PK digested brain homogenate from cases of inherited prion disease with *PRNP* mutations showing protease-resistant PrP fragments of ~6–8 kDa. The *PRNP* point mutation is designated above each immunoblot. Immunoblots were developed with anti-PrP monoclonal antibody 3F4.

Fig. 5. PrP glycoform ratios in human prion disease. PK digestion of brain homogenate and analysis by enhanced chemifluorescence with anti-PrP monoclonal antibody 3F4 enables calculation of the proportions of di-, mono-, and nonglycosylated PrP. The plot shows the protease-resistant PrP glycoform ratio seen classical CJD (PrP^{Sc} types 1–3), vCJD (PrP^{Sc} type 4 in brain and type 4t PrP^{Sc} in tonsil) and in cases of inherited prion

disease. The key shows PrP^{Sc} type or mutation and *PRNP* codon 129 genotype (methionine [M] and valine [V]). Classification according to Hill et al. ***(9, 10)***. Data points represent the mean relative proportions of di- and mono-PrP as percentage ± S.E.M. In some cases the error bars were smaller than the symbols used.

Fig. 6. Prion disease pathology. Brain sections from sCJD and vCJD show spongiform neurodegeneration after hematoxylin and eosin staining (H&E), proliferation of reactive astrocytes after immunohistochemistry using anti-GFAP antibodies (GFAP), and abnormal PrP immunoreactivity after immunohistochemistry using anti-PrP monoclonal antibody ICSM35 (PrP). Abnormal PrP deposition in sCJD most commonly presents as diffuse, synaptic staining, whereas vCJD is distinguished by the presence of florid PrP plaques consisting of a round amyloid core surrounded by a ring of spongiform vacuoles. Bar = 100 μm. Inset, high-power magnification of a florid PrP plaque.

Fig. 7. Abnormal PrP in vCJD tonsil and appendix. **(A)** Diagnostic PrP^{Sc} analysis of tonsil biopsy tissue. Aliquots (0.5 ml) of 10% (w/v) tonsil biopsy homogenate from a patient with suspected vCJD or 10% normal human tonsil homogenate, either lacking or containing a spike of 50 nl of 10% (w/v) vCJD brain homogenate, were subjected to sodium phosphotungstic acid precipitation. Then, 20 μl aliquots of whole samples isolated before centrifugation were analyzed in the absence of PK digestion (–) and compared with PK digestion products (+) derived from the entire sodium phosphotungstic acid pellets. The immunoblot was analyzed with anti-PrP monoclonal antibody 3F4 and high-sensitivity enhanced chemiluminescence. **(B)** PK-digested sodium

phosphotungstic acid pellet derived from 0.5 ml 10 % (w/v) appendix homogenate from a patient with neuropathologically confirmed vCJD analyzed by high-sensitivity enhanced chemiluminescence using anti-PrP monoclonal antibody 3F4 **(C)**. Immunohistochemical analysis of vCJD tonsil **(i)** and appendix **(ii)** obtained at autopsy. Abnormal PrP immunoreactivity is confined to lymphatic follicles with deposition mainly in dendritic cells. Anti-PrP monoclonal antibody ICSM 35. Bar = 160 μm (C,i) and 100 μm (C, ii). Insets, high-power magnification of PrP deposits.

Table 1

Protocol for overnight processing of tissue samples for immunohistochemistry

Solution	Time (min)	Temperature
10% buffered formal-saline	30	Ambient
IMS 70%	75	Ambient
IMS 70%	75	Ambient
IMS 70%	75	Ambient
IMS 90%	60	Ambient
IMS 90%	60	Ambient
IMS 100%	75	Ambient
IMS 100%	75	Ambient
Xylene	75	Ambient
Xylene	75	Ambient
Molten paraffin wax	50	60°C
Molten paraffin wax	50	60°C
Molten paraffin wax	50	60°C
Molten paraffin wax	50	60°C

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