Supporting Information

Determining the relative susceptibility of four genotypes to atypical scrapie.

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Mass Spectrometry.

The instrument response was optimized for each peptide by a previously described method [1]. The mass spectrometer was operated in multiple reaction monitoring (MRM) mode, alternating between detection of the six sheep peptides and their ^{15}N-labeled internal standards. The mass settings for the sheep peptides are summarized in Table S-1.

Poly	Type	Parent Ion		Product Ion	Ion	Q1	Q3	time (ms)	DP	EP	CE	CXP
Octarepeat (61,69,77)	^{14}N	GQPHGGGW		PHGGGW	y6	398.18	610.27	50	91.3	4.1	19.1	15
Octarepeat (61,69,77)	^{14}N	GQPHGGGW		GQPHGGG	b7	398.18	591.26	50	91.3	4.1	20.1	15
Octarepeat (61,69,77)	^{15}N	GQPHGGGW		PHGGGW	y6	404.16	619.25	50	91.3	4.1	19.1	15
Octarepeat (61,69,77)	^{15}N	GQPHGGGW		GQPHGGG	b7	404.16	601.23	50	91.3	4.1	20.1	15
F_{141}	^{14}N	MLGSAMSRP**F**		MLGSAMSR	b8	548.77	834.4	50	97	5.8	22.8	15
F_{141}	^{15}N	MLGSAMSRP**F**		MLGSAMSR	b8	555.25	845.4	50	97	5.8	22.8	15
F_{141}	^{14}N	MLGSAMSRP**F**		GSAMSRP**F**	y8	548.77	852.4	50	97	5.8	27.9	15
F_{141}	^{14}N	M(ox)LGSAMSRP**F**		GSAMSRP**F**	y8-ox	556.8	852.4	50	97	5.8	27.9	15
F_{141}	^{14}N	MLGSAM(ox)SRP**F**		GSAM(ox)SRP**F**	y8-ox	556.8	868.4	50	97	5.8	27.9	15
F_{141}	^{14}N	M(ox)LGSAM(ox)SRP**F**		GSAM(ox)SRP**F**	y8-ox	564.8	868.4	50	97	5.8	27.9	15
F_{141}	^{15}N	MLGSAMSRP**F**		GSAMSRP**F**	y8	555.25	863.4	50	97	5.8	27.9	15
F_{141}	^{15}N	M(ox)LGSAMSRP**F**		GSAMSRP**F**	y8-ox	563.2	863.4	50	97	5.8	27.9	15
F_{141}	^{15}N	MLGSAM(ox)SRP**F**		GSAM(ox)SRP**F**	y8-ox	563.2	879.4	50	97	5.8	27.9	15
F_{141}	^{15}N	M(ox)LGSAM(ox)SRP**F**		GSAM(ox)SRP**F**	y8-ox	571.2	879.4	50	97	5.8	27.9	15
D_{172}	^{14}N	YRPVDQ**D**SNQNNF		PVDQ**D**SNQNNF	y11	798.86	1277.5	50	116.8	10.8	33.2	15
D_{172}	^{14}N	YRPVDQ**D**SNQNNF		YRPVDQ**D**SNQNN	b12 2+	798.86	716.32	50	116.8	10.8	34.5	15
D_{172}	^{14}N	YRPVDQ**D**SNQNNF		YRPVDQ**D**	b7	798.86	874.41	50	116.8	10.8	49.12	15
D_{172}	^{14}N	YRPVDQ**D**SNQNNF		YRPVD	b5	798.86	631.32	50	116.8	10.8	57.8	15
D_{172}	^{15}N	YRPVDQ**D**SNQNNF		PVDQ**D**SNQNNF	y11	809.32	1293.5	50	116.8	10.8	33.2	15
D_{172}	^{15}N	YRPVDQ**D**SNQNNF		YRPVDQ**D**SNQNN	b12 2+	809.32	726.29	50	116.8	10.8	34.5	15
D_{172}	^{15}N	YRPVDQ**D**SNQNNF		YRPVDQ**D**	b7	809.32	885.37	50	116.8	10.8	49.12	15
D_{172}	^{15}N	YRPVDQ**D**SNQNNF		YRPVD	b5	809.32	639.3	50	116.8	10.8	57.8	15

Table S-1. A table of optimized parent ions, product ions and their corresponding optimized parameters. Declustering potential (DP), entrance potential (EP), collision energy (CE) and collision exit potential (CXP) for the listed three sheep chymotryptic peptides derived from two sheep PrP polymorphisms (Poly). The polymorphisms are indicated in **bold underline**.

Supporting Experimental Procedures (Materials and Methods)

Chemicals. LC-MS grade water and acetonitrile, dithiothreitol, guanidine hydrochloride, and sarkosyl were purchased from Fisher Scientific (Pittsburgh, PA). Chymotrypsin was purchased Worthington Biochemical Corporation (Lakewood, NJ). The GE8 antibody was purchased from the TSE Resource Centre (now in the Roslin Institute, Scotland, UK). The L42 mAb was purchased from R-Biopharm, Inc. (Washington, MO). Anti-Mouse IgG (Fc specific)–AP (goat anti-mouse), was purchased from Sigma-Aldrich (St. Louis, MO). The MES running buffer and LDS loading buffers were obtained from Thermo Fisher Scientific (Waltham, MA). PVDF Membranes (0.2mm pore size) and thick blotting paper were obtained from Bio-Rad (Hercules, CA). Novex NuPAGE 4-12% Bis-Tris Gels, either 1.5 mm x 10 well or 1.0 mm x 10 well were obtained from Thermo Fisher Scientific (Waltham, MA). StartingBlock T20 (PBS) blocking buffer was obtained from Thermo Fisher Scientific (Waltham, MA). All other reagents were from Sigma-Aldrich (St. Louis, MO).

Animal handling and tissue samples. The tissues used in this study were harvested from sheep naturally infected with atypical scrapie (Table S-2). The tissue was collected from fallen stock, animals sent to a slaughterhouse for human consumption, or control animals. The tissue was divided into three parts, one portion was fixed for immunohistochemical analysis (IHC), another was used for ELISA analysis, and a small portion was frozen for future mass spectrometry-based analysis. These animals were identified as part of a routine screening for small ruminant TSEs [2]. Uninfected brain tissue came from heterozygous, humanely euthanized animals that tested negative for classical or atypical scrapie. The brain tissue samples from the infected animals came from the brainstem and the samples from the uninfected animals came from either the cerebellum or cranial spinal cord. The cases of atypical scrapie were confirmed by Western blot (WB), using the BioRad or Idexx test, and IHC, using L42 (epitope: Y_{148}EDRYY_{153}) and F89 (epitope: I_{142}HFG_{145}) monoclonal antibodies [3, 4].

Genotypes of uninfected sheep and sheep infected with atypical scrapie. The two uninfected sheep possessed the $A_{136}L_{141}R_{154}R_{171}Y_{172}/A_{136}L_{141}R_{154}Q_{171}Y_{172}$ or $A_{136}L_{141}R_{154}R_{171}Y_{172}/A_{136}L_{141}H_{154}Q_{171}Y_{172}$ genotype (Table S-2 and Figure S-1). Two infected sheep possessed the $A_{136}L_{141}R_{154}R_{171}Y_{172}/A_{136}L_{141}H_{154}Q_{171}Y_{172}$ genotype and silent polymorphisms at positions 231 (agg/cgg) and 237 (ctc/ctg). The other two animals infected with atypical scrapie had the $A_{136}L_{141}R_{154}R_{171}Y_{172}/A_{136}F_{141}R_{154}Q_{171}Y_{172}$ or $A_{136}L_{141}R_{154}R_{171}Y_{172}/A_{136}L_{141}R_{154}Q_{171}D_{172}$ genotype with no silent polymorphisms (Table S-2 and Figure S-1).

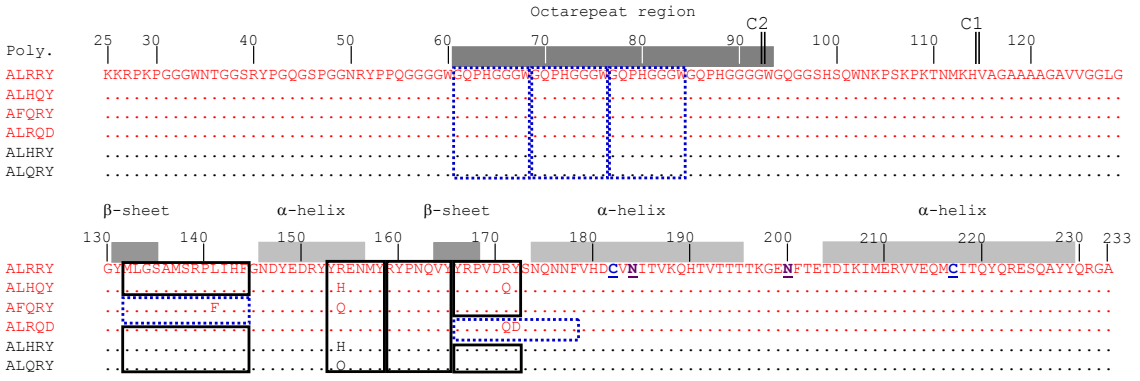


Figure S-1. Sequences of sheep PrP^{C} polymorphisms (Poly.) associated with atypical scrapie.[5] Polymorphisms at positions 136/141/154/171/172 of sheep PrP are summarized by a five-letter shorthand indicating the amino acid present at that position. The sheep PrP, ALRRY, possesses A at position 136, L at 141, R at 154, R at 171, and Y at 172. Relevant primary and secondary structures are indicated. The location of the two cysteines (C) forming the disulfide bond and the sites of the asparagine (N) linked glycosylation are indicated. The sequences used in this work are indicated in red. Chymotryptic peptides used to identify the relevant polymorphisms are defined by solid or dotted rectangles. Those bounded by blue dotted rectangles are described in this work, while those bounded by black solid rectangles are described in previous work. The C1 and C2 cleavage sites are indicated by the double lines.[6, 7] A 24-mer signal peptide (MVKSHIGSWILVLFVAMWSDVGLC) directing the protein to the endoplasmic reticulum is cleaved from the nascent peptide, hence the numbering starting with 25.[8]

Infected and control sheep samples. Atypical scrapie was identified in four heterozygous sheep, by immunohistochemistry (IHC) and ELISA-based tests. The genotypes of these animals were determined by sequencing the PrP gene. In addition, brain tissue from two uninfected control animals was analyzed for comparison purposes. These data are summarized in Table S-2. IHC slides are shown in Figure S-2. Western blot-based analysis on O-1359 and O-1376 is shown in Figure S-3.

Animal #	Genotype (136/141/154/171/172)	Tissue	ELISA result	IHC result	Animal source
O-1359	AL**RR**Y/AL**HQ**Y	Brainstem	1.923* (medium +)	L42, F89 + MO +++CBLO	Slaughterhouse (humans food chain)
O-1376	AL**RR**Y/AL**HQ**Y	Brainstem	3.187♦ (high +)	L42, F89 ++	Fallen stock
O-1391	ALR**RY**/ALR**QD**	Brainstem	0.375* (low +)	L42 +, F89 +	Slaughterhouse (humans food chain)
O-1800	A**L**RR**Y**/A**F**R**Q**Y	Brainstem	1.125* (medium +)	L42, F89 ++	Fallen stock
O-1256	AL**RR**Y/AL**HQ**Y	Cranial spinal cord	Negative	Negative	Control
O-1388(s)	ALR**R**Y/ALR**Q**Y	Cranial spinal cord	Negative	Negative	Control
O-1388(c)	ALR**R**Y/ALR**Q**Y	Cerebellum	Negative	Negative	Control

Table S-2. Summary of the ELISA, immunohistochemistry (IHC) and other data related to the samples. Samples 1256 and 1388 are uninfected controls. * BioRad test; ♦ Idexx test.

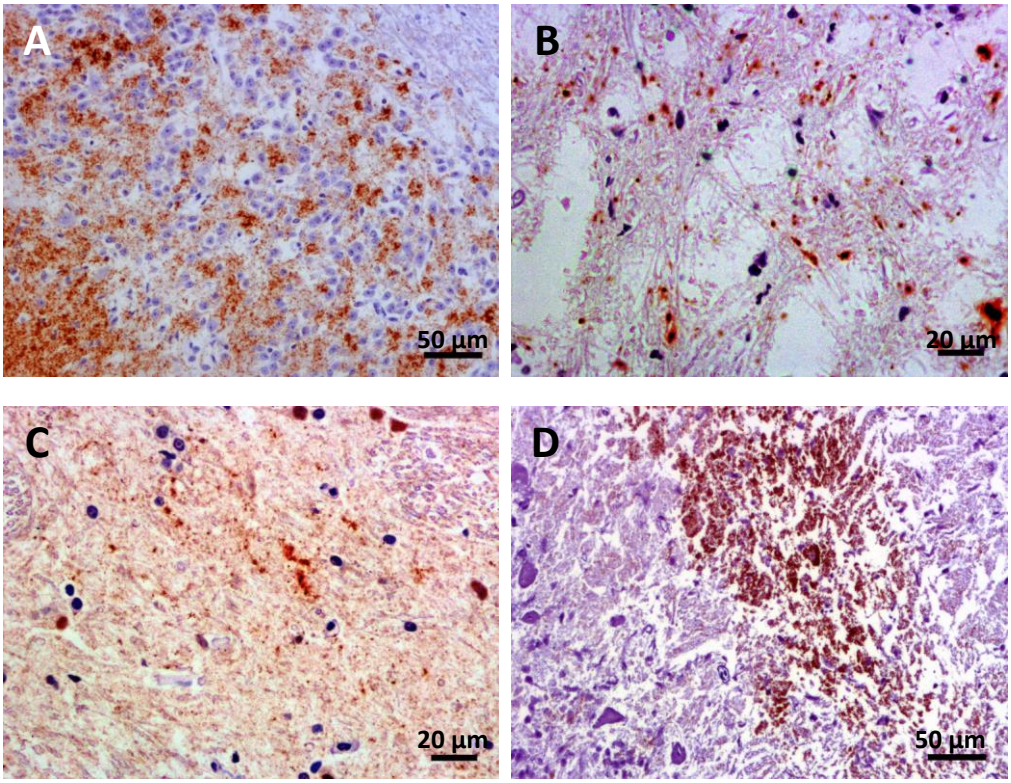
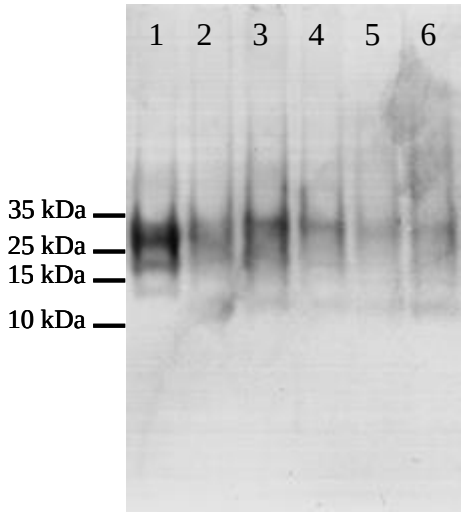


Figure S-2. Detection of PrP^{Sc} by immunohistochemical techniques using the L42 monoclonal antibody.[9] 3,3′-Diaminobenzidine (DAB) stained (brown) section from the cerebellum of O-1359 (A). DAB stained section from the obex of O-1376 (B). DAB stained section from the obex of O-1391 (C). DAB stained section from the cerebellum of O-1800 (D).



Lane	Sample
1	Classical scrapie
2	Atypical scrapie
3	Atypical scrapie
4	Atypical scrapie
5	Sample O-1359
6	Sample O-1376

Figure S-3. Western blot of PK treated atypical scrapie samples. Each sample is a proteinase K treated pellet (5 μg/mL of proteinase K for 30 minutes at 37 °C) from an ultracentrifugation (*vide infra*) of 500 μL of a 10% brain homogenate. Lane 1 is from a case of classical scrapie. Lanes 2-4 are from sheep experimentally infected with atypical scrapie. Lane 5 is sample O-1376. Lane 6 is sample O-1359. The limited amounts of O-1391 and O-1800 precluded their analysis by Western blot. The PK-treated atypical scrapie samples (lanes 2-6), show a characteristic band (~ 12 kDa) that is absent in the PK-treated classical scrapie sample (Lane 1).

Isolation of PrP^{Sc}.

PrP^{Sc} was isolated according to the methods of Bolton *et al*. with some minor modifications [10, 11]. Briefly, a 10% tissue homogenate (w/v), from the spinal cord near the brain, or from the brain, was made using an Omni GLH general laboratory homogenizer and disposable Omni Tip plastic generator probes, in homogenization buffer (10% w/v N-Lauroylsarcosine sodium salt, 9.5 mM sodium phosphate, pH 8.5) and allowed to stand for 30 minutes at room temperature. The homogenate was then centrifuged for 18 minutes (16,000 x *g*; 20ºC), in a refrigerated centrifuge (Eppendorf 5810R), to remove large particles. The supernatant was retained. Five hundred microliter aliquots of each tissue homogenate were separately diluted to 3 ml with homogenization buffer and individually transferred to a separate ultracentrifuge tube (4.2 mL, 16 x 38 mm).

The homogenization buffer in each tube was underlaid with 1 ml of 20% w/v sucrose and sealed. Each sealed tube was centrifuged for 75 minutes at 150,000 x *g* (46,000 rpm, 20 ºC) in a Beckman 70.1 Ti rotor to obtain a PrP^{Sc} containing pellet. Each pellet was individually denatured by the addition of 200 μL of 8M guanidinium chloride (GuCl). Each solution was allowed to stand for 24 hours at room temperature. The denatured PrP protein was precipitated with ice-cold methanol (85% methanol to

15% protein solution) and centrifuged for 20 minutes at 20,000 x *g* for 20 min in a cold rotor (-9 °C) with an Eppendorf Model 5417R centrifuge (Hamburg, Germany). The pellet resulting from the methanol precipitation was subjected to reduction, alkylation and chymotryptic cleavage (*vide infra*).

Proteinase K treatment of samples. Five hundred μL of a 10% brain homogenate from either a sheep naturally infected with classical scrapie, or three different samples of sheep infected with atypical scrapie, or sample O-1376 or sample O-1359, were separately subjected to the method of Bolton *et al.* (*vide supra*) to isolate the PrP^{Sc} present in these six samples. Each pellet was separately resuspended in 100 μl of digest buffer (0.1 % w/v 3-(*N*,*N*-Dimethylmyristylammonio)propanesulfonate, 20 mM Tris, pH 8.5) by brief sonication (four 45-second bursts; Misonix 4000 sonicator). Enough PK (100 μg/mL) was added to each of the six solutions make a final concentration of 5 μg/mL PK. The PK-containing solutions were allowed to digest for 30 minutes at 37 °C. After the digestion was completed one μL of a 100 mg/mL solution of Phenylmethylsulfonyl fluoride (PMSF) was added to stop the reaction. The samples were denatured by the addition of 300 μL of 8M GuCl and allowed to stand overnight. The samples were methanol precipitated and then brought up in 4 x LDS. The samples were analyzed by Western blot (*vide infra*) using L42 as the primary antibody (Figure S-3).[12]

Isolation of PrP^{C}. A HisPur Ni-NTA column, where the divalent nickel was replaced by divalent copper, was prepared as follows. The column was washed with 10 mL of water (1 mL per minute) and then washed (1 mL/minute) with 10 mL of stripping buffer (100 mM EDTA; pH 8.0) to remove the Ni^{2+}. The stripped column was washed with 10 mL of water (1 mL/ minute) and then charged (1 mL/minute) with 3 mL of 0.5 M $CuSO_4$. The Cu^{2+} column was washed with 10 mL of water to removed the excess $CuSO_4$ and then equilibrated (1 mL/minute) with 10 mL of buffer (5% sarkosyl in 4M GuCl). The column was ready for use in the following purification.

The supernatants from individual Bolton preparations (*vide supra*) were separately diluted with an equivalent volume of 8M GuCl, mixed, and allowed to stand for 24 hours a room temperature [13]. Each sample (25 mg brain tissue in 4M GuCl and 5 % sarkosyl) was loaded onto a separate Cu^{2+} column at a flow rate of 1 mL/minute. The column was sequentially eluted (1 mL/minute) with the listed 10 mL solutions: 4M GuCl, 20 mM imidazole in 4 M GuCl, 100 mM imidazole in 4 M GuCl, 250 mM imidazole and 5 % sarkosyl in 2 M GuCl, and 100 mM EDTA (pH 8.0) and 5 % sarkosyl in 2 M GuCl. Four mL of each fraction was methanol precipitated (vide supra) for either mass spectrometry-based analysis, Western blot, or Coomassie stained SDS-PAGE.

Reduction, alkylation and chymotryptic cleavage of PrP samples. Each pellet derived from methanol precipitation (*vide supra*) or aliquot of recombinant PrP was dissolved in 20 μl of buffer (0.01% β-octylglucopyranoside (BOG), 1 pmol/μl methionine, and 8% acetonitrile) and sonicated (Cole-Parmer model 8892; Vernon Hills, IL) for 5 minutes. A 10 μl aliquot of 15mM dithiothreitol (DTT) in buffer A (25 mM ammonium bicarbonate, 0.01% β-octylglucopyranoside (BOG), 1 pmol/μl methionine, and 8% acetonitrile; pH 8.6) was added to the sonicated solution and reacted for 1 hr (37 ºC). The reaction mixture was cooled to room temperature and then 40 μl of iodoacetamide (IA) buffer (22

mM iodoacetamide in buffer A) was added to the mixture and left to react in the dark at room temperature for 1 hour. Any excess IA was quenched by the addition of 20µl of DTT buffer (22 mM DTT in buffer A). The reduced and alkylated proteins were subjected to proteolysis with added chymotrypsin (500 ng chymotrypsin/10 µl of a 100 mM $CaCl_2$ solution). The chymotrypsin reaction was allowed to proceed at 30 ºC for 20 hours. After the digestion was complete, the chymotryptic digestion was stopped by the addition of 2.5 µl of 10% formic acid. Each sample was filtered through a 10,000 molecular weight cutoff filter (VWR International, San Francisco, CA) for 12 minutes at 14,000 x g. Samples were stored at -20 ºC until analyzed. There were either two or four biological replicates for each sample (Bolton pellets). There were three technical replicates (individual injections on the mass spectrometer) for each biological replicate.

Qualitative Mass Spectrometry. The qualitative mass spectrometry was performed using a Thermo Scientific, Orbitrap Elite mass spectrometer (Thermo Scientific, Waltham, MA). Synthetic peptides or peptides generated from recombinant proteins digested with chymotrypsin, were solubilized with 2% acetonitrile, 0.1% formic acid in water (Optima LC/MS grade, Fisher Scientific, Pittsburgh, PA), to approximately 1 pmol/uL. Each sample was transferred to an autosampler vial and placed in the autosampler of an EASY-nLC II interfaced to an Orbitrap Elite mass spectrometer with a PicoChip nanospray source (New Objective, Woburn, MA). For each LC-MS run, a 4uL portion of sample was loaded by the autosampler onto a 75 µm ID column containing 10 cm of 3 µm, 120 Å, ReproSil-Pur C18-AQ reverse phase packing (New Objective, Woburn, MA).

Samples were eluted into the mass spectrometer with a binary gradient flow at a rate of 400 nL/minute. Solvent A was 2% acetonitrile in 0.1% formic acid, and Solvent B was acetonitrile in 0.1% formic acid (Optima LC/MS grade; Fisher Scientific, Pittsburgh, PA). The gradient was programmed from 5% Solvent B to 35% Solvent B over 45 minutes, held at 35% B for 13 minutes, then returned to 5% B for 2 minutes. Peptides were detected in the Orbitrap with the FT survey scan from 300 to 2000 m/z at a resolution of 60,000. The 10 most intense peaks above a threshold of 30,000 counts were subject to dissociation (CID) with normalized collision energy set to 35, default charge state set to 2, isolation width set to 2.0 m/z, and activation time set to 30 ms. Dynamic exclusion was enabled for duration of 6 seconds with a repeat count of 1. Charge state screening allowed + 1 and greater charge states to be selected for CID fragmentation. Monoisotopic precursor selection was enabled. MS/MS data were searched against both public and in-house generated databases using the Mascot Server protein identification software (Matrix Science Inc., Boston, MA) to identify peptides of interest.

Quantitative Mass Spectrometry: Nanospray LCMSMS. An Applied Biosystems (ABI/MDS Sciex, Toronto, ON) model 4000 Q-Trap instrument equipped with a nanoelectrospray source was used to perform nanospray liquid chromatography and tandem mass spectroscopy (LC-MS/MS). An aliquot (6 µL) of each digest with ^{15}N-labeled internal standard was loaded onto a C-18 trap cartridge [Acclaim PepMap100, 5 µm, 100Å, 300 µm (inside diameter) x 5 mm (Dionex, Sunnyvale, CA)]. Salts were washed from the cartridge with an acetic acid/acetonitrile/heptafluorobutyric acid/water solution (0.5/1/0.02/99). The now salt-free bound peptides were eluted onto a reverse

phase column [Vydac Everest (Hichrom; Reading, UK), 75 μm x 150 mm]. The solvents were delivered with an Applied Biosystems model Tempo nanoflow LC system (ABI/MDS Sciex) with an autosampler, a column switching device, and a nanoflow solvent delivery system. Samples were eluted from the column with a binary gradient (A, 2% acetonitrile with 0.5% acetic acid in water, and B, 80% acetonitrile with 0.5% acetic acid). The flow rate was 310 nL/min with a 20 min linear gradient starting with 5% B and ending with 30% B, followed by a 5 min linear gradient from 30% to 100% B. Elution with 100% B was conducted for 60 min followed by a return to 5% B over 5 min. The column was equilibrated for 30 min at 5% B. The eluted samples were sprayed with a non-coated spray tip (FS360-20-10-N-20-C12, New Objective Inc., Woburn, MA) onto the Applied Biosystems source, Model Nanospray II.

The mass spectrometer was operated in multiple reaction monitoring (MRM) mode, alternating between detection of the analyte peptides and their appropriate ^{15}N-labeled internal standards. The mass settings (*vide supra*) for the chymotryptic peptides were empirically determined and may be found in the Supporting Information. The mass settings for the quantification were done with the IntelliQuant quantification algorithm using Analyst 1.5 software.

Preparation of the polymorphisms at positions 141 and 172 in sheep recombinant PrP. The sheep Prnp corresponding to amino acids 25-233 (lacking the N- and C-terminal signal sequences; $A_{136}L_{141}R_{154}Q_{171}Y_{172}$) was used for site specific mutagenesis. The polymorphisms at positions 141 and 172 were generated using the standard megaprimer method of site directed mutagenesis [14, 15]. Briefly, two rounds of PCR were performed, the first to generate the megaprimer containing the mutation of interest, and the second to amplify the whole sequence including restriction sites for cloning. All PCR reactions were performed using Phusion® Hot Start Flex DNA Polymerase (New England Biolabs, Ipswich, MA) and standard reagents at the recommended concentrations and using the cycles advised for the enzyme. The two rounds of PCR required the synthesis of two DNA primers (Elim Biopharmaceuticals, Hayward, CA). The forward mutagenic primers for F_{141} and D_{172} polymorphisms used in the first round of PCR are listed as follows:

F_{141}: 5'- GTCATGAGCAGGCCTTTTATACATTTTGG –3'
D_{172}: 5'- GACCAGTGGATCAGGATAGTAACCAGAACAAC –3'

The F_{141} and D_{172} polymorphisms were generated by using the F_{141} and D_{172} primer combined with the ARQ polymorphism as the template. The mutagenic primers above were used with a vector derived reverse primer (pET-hiTm2; GCTAGTTATTGCTCAGCGGTGGC) to generate the megaprimer. The PCR product containing the megaprimer was diluted 25x and used in the next round of PCR with the following forward primer:

Sheep-F-Nde: AACACACCCATATGAAGAAGCGACCAAAACCTGG

Products were checked for correct length by gel, then purified and digested with NdeI and BamHI and ligated into a pET11a vector (also digested and treated with phosphatase). The ligation mixture (3:1 molar ratio of insert:vector) was transformed into chemically competent DH5α cells (New England Biolabs, Ipswich, MA). The inserts were sequenced with an Applied Biosystems 3730 Genetic Analyzer (Applied

Biosystems, Foster City, CA) to verify each mutation. The correctly mutated, sequenced plasmids were isolated and transformed into BL21 cells (EMD Millipore, Billerica, MA) for expression.

Analysis of the GQPHGGGW peptide. Sheep PrP possesses three copies of GQPHGGGW as part of a copper binding motif of PrP^C referred to as the octarepeat region (Figure S-1). We have previously identified this peptide in chymotryptic digests of sheep rPrP. ^{15}N-labeled GQPHGGGW is present in the chymotryptic digests of ^{15}N-labeled sheep rPrP, so these digests can also be used to generate an internal standard for this peptide. GQPHGGGW was chemically synthesized (> 95% pure) and used to optimize instrument parameters (Table S-1). The presence of the other chymotryptic (^{14}N- or ^{15}N-labeled) peptides did not interfere with the analysis of this peptide. A calibration curve relating the area ratio of the signal for the GQPHGGGW peptide to that of $R_{159}YPNQVY_{165}$ was prepared. The relationship is linear with an excellent correlation coefficient (> 0.999; Figure S-8).

Preparation of the natural abundance or ^{15}N-labeled sheep PrP.

Each of the BL21 clones was grown in M9 minimal medium (84.5mM Na_2HPO_4, 44.4mM KH_2PO_4, 17.1mM NaCl, 37.4mM NH_4Cl, 2mM $MgSO_4$, 0.1mM $CaCl_2$, 33.2μM thiamine, 22.2mM glucose, and trace metals) supplemented with either natural abundance NH_4Cl or $^{15}NH_4Cl$ (99.7% ^{15}N). Twenty-five ml of the appropriate M9 medium (supplemented with 100 μg/mL of carbenicillin) in 250 mL flasks was inoculated with a single colony of the desired clone ($A_{136}\mathbf{F}_{141}R_{154}Q_{171}Y_{172}$ and $A_{136}L_{141}R_{154}Q_{171}\mathbf{D}_{172}$) and allowed to grow overnight in a shaker/incubator (250 rpm; 37 °C). In order to minimize isotopic dilution of ^{15}N, four microliters of the overnight cultures grown in M9 medium (+ $^{15}NH_4Cl$) were inoculated into a fresh 25 mL culture of M9 medium supplemented with $^{15}NH_4Cl$ and allowed to grow overnight. Four mL of the appropriate overnight culture was added to 150 mL of fresh M9 medium (supplemented with 100 μg/mL of carbenicillin and either natural abundance NH_4Cl or $^{15}NH_4Cl$) in a 1 L flask and allowed to grow until the cells achieved mid log growth (A_{600} between 0.4 and 0.6). Once mid log growth was achieved, the cells were induced to overexpress the cloned protein by the addition of a sufficient amount of Isopropyl β-D-1-thiogalactopyranoside (IPTG) to make a 1 mM solution. The cells were allowed to grow for four more hours. The cells were pelleted (10,000 x g; 15 min), washed, and then pelleted again. The inclusion bodies were isolated and rPrP purified by previously described methods [11].

The isolated inclusion bodies from the cells grown in $^{15}NH_4Cl$ were isolated, denatured, slowly renatured on an immobilized metal affinity column, and then chromatographed on that column. The fraction containing the ^{15}N-labeled PrP was dialyzed against ammonium acetate (50 mM, pH 4.5) to remove the non-volatile salts. The retentate was lyophilized to yield a white powder.

The powder was dissolved in buffer (0.01% β-octylglucopyranoside (BOG), 1 pmol/μl methionine, and 8% acetonitrile) and then reduced, alkylated, and digested with chymotrypsin. The resulting ^{15}N-labeled peptides were used as internal standards.

Western blots. The SDS-PAGE gels (1.5 mm thick, 10 well; NuPAGE 4-12% Bis-Tris) were purchased from a commercial vendor and used according to the manufacturer's instructions. The resulting gels, thick blotting paper and PVDF membrane were soaked in transfer buffer (25 mM Tris, 250 mM glycine in 15% methanol) for 10 minutes prior to transfer (BIO-RAD Trans-Blot SD semi-dry transfer cell (Hercules, CA); 50 minutes at 100 mA). Upon completion of the transfer, the PVDF membrane was blocked (10 mL of StartingBlock T20; 15 minutes RT). After blocking, the membrane was incubated with the primary antibody (GE8 or L42 mAb) (5 microliters (GE8) or 10 microliters (L42) of a 1 mg/mL stock in 10 mL of blocking buffer) and gently rotated for 1 hour at RT. After the incubation, the membrane was transferred into a fresh dish and washed with 20 mL of wash buffer (PBS + 25 μL Tween 20). The wash was discarded and the membrane washed a further 3 more times under identical conditions. Upon completion of the wash steps, 10 mL of blocking buffer containing 10 μL of the secondary antibody, AP-Goat Anti-Mouse IgG (Fc), was added. The membrane was gently rotated at room temperature for 45 minutes. After 45 minutes, the membrane was transferred into a new container and washed four times at five minute intervals.

After the washing was complete the blot was placed in 10 mL of a Sigma/FAST BCIP/NBT solution (1 tablet per 10mL per manufacturer's instructions) and incubated at 37 °C. After development, the blots were dried and scanned.

Coomassie stained gel. The samples were loaded onto a SDS-PAGE gel (1.0 mm thick, 10 well; NuPAGE 4-12% Bis-Tris) obtained from a commercial vendor. After the run was completed, the gel was stained with a Coomassie rapid stain with 10 sec of microwaving followed by 30 min of incubation. The gel was destained until the background was only very light blue. The gel was scanned.

Safety considerations.

This BSL2 laboratory was certified and inspected by the Animal and Plant Health Inspection Service (APHIS) of the USDA (www.aphis.usda.gov/permits/). Proper BSL2 procedures are outlined in the 5th edition of the CDC's biosafety manual, Biosafety in Microbiological and Biomedical Laboratories [16]. Scrapie was inactivated before removal from the dedicated BSL-2 laboratory by the addition of a sufficient volume of 8M guanidinium chloride to make a 6M or 4M solution. The solution was thoroughly mixed and then allowed to stand for at least 24 hours at room temperature to ensure that the prions were inactivated [13]. The solution of inactivated prions was transferred to a clean fresh tube and removed from the BSL-2 laboratory. After inactivated prions were digested with proteases, they were filtered though a 10 kDa MWCO filter, before they were analyzed by a mass spectrometer.

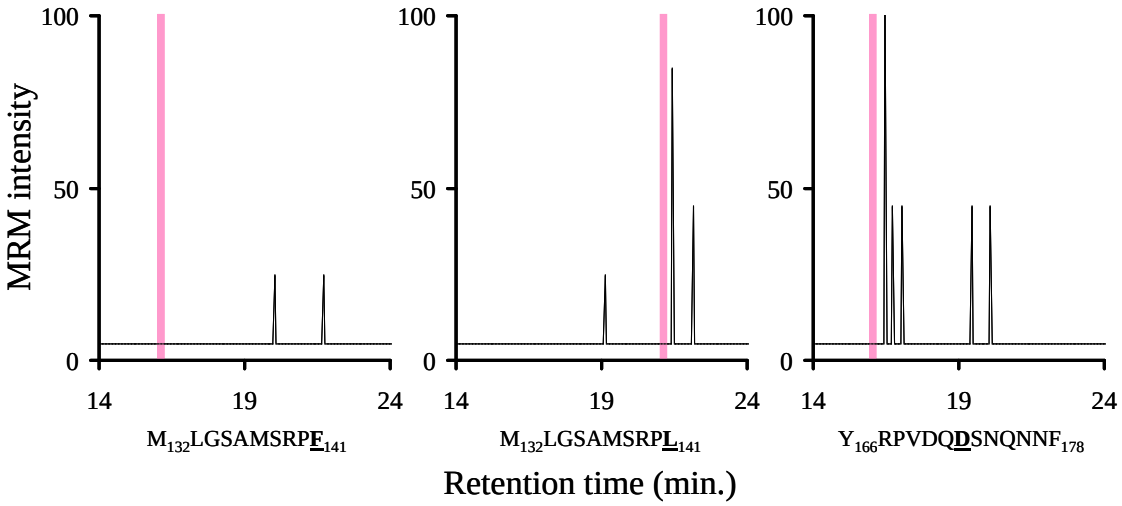


Figure S-4. Chromatograms of molecules present in the chymotryptic digest of a Bolton pellet isolated from a homozygous ($A_{136}L_{141}R_{154}Q_{171}Y_{172}/A_{136}L_{141}R_{154}Q_{171}Y_{172}$) uninfected sheep brain homogenate. Each is an MRM-based chromatogram, using the parameters for the indicated chymotryptic peptide polymorphism. Pink shaded columns indicate expected retention times of signals for the respective peptide polymorphism.

Calibration curves (Figure S-5 to S-8)

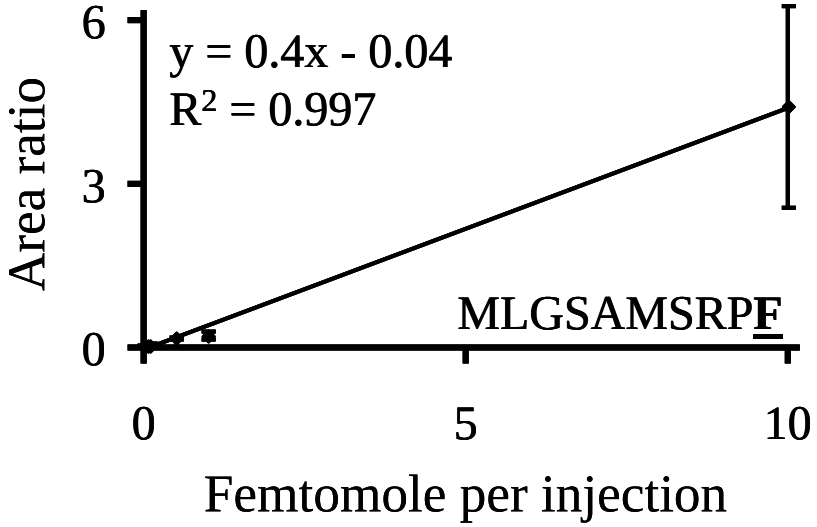


Figure S-5. Calibration curves for the sheep peptide, M_{132}LGSAMSRP**F**$_{141}$. This calibration curve was used to determine the empirical relationship between the concentration of the M_{132}LGSAMSRP**F**$_{141}$ peptide and the R_{159}YPNQVY$_{165}$ peptide used as an internal standard. All concentrations are reported as the mean ± standard deviation of four injections.

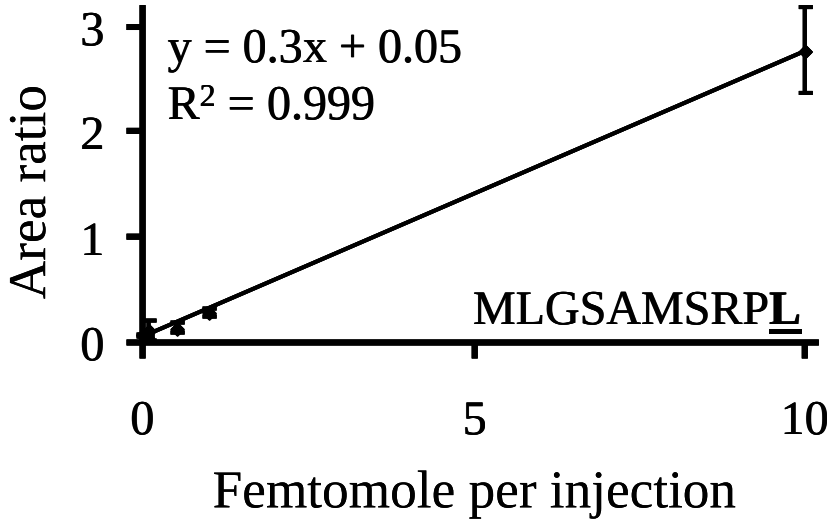


Figure S-6. Calibration curves for the sheep peptide, M_{132}LGSAMSRP**L**$_{141}$. This calibration curve was used to determine the empirical relationship between the concentration of the M_{132}LGSAMSRP**L**$_{141}$ peptide and the R_{159}YPNQVY$_{165}$ peptide used as an internal standard. All concentrations are reported as the mean ± standard deviation of four injections.

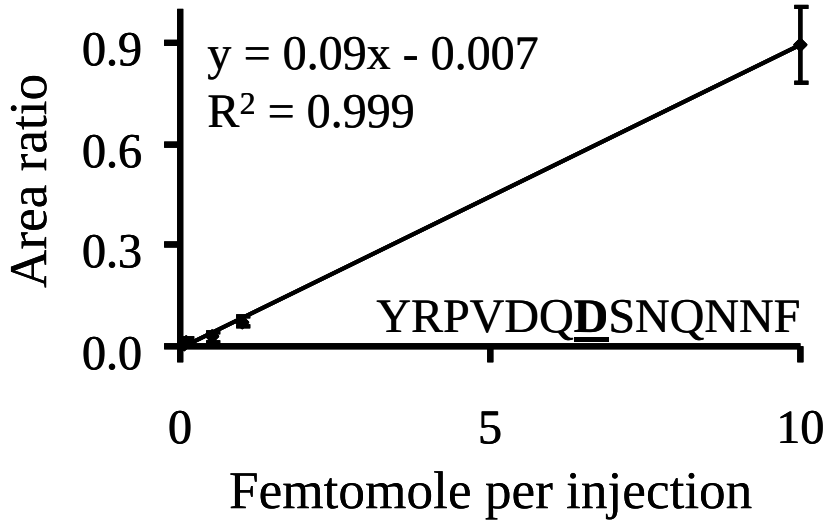


Figure S-7. Calibration curves for the sheep peptide, Y_{166}RPVDQ**D**SNQNNF$_{178}$. This calibration curve was used to determine the empirical relationship between the concentration of the Y_{166}RPVDQ**D**SNQNNF$_{178}$ peptide and the R_{159}YPNQVY$_{165}$ peptide used as an internal standard. All concentrations are reported as the mean ± standard deviation of four injections.

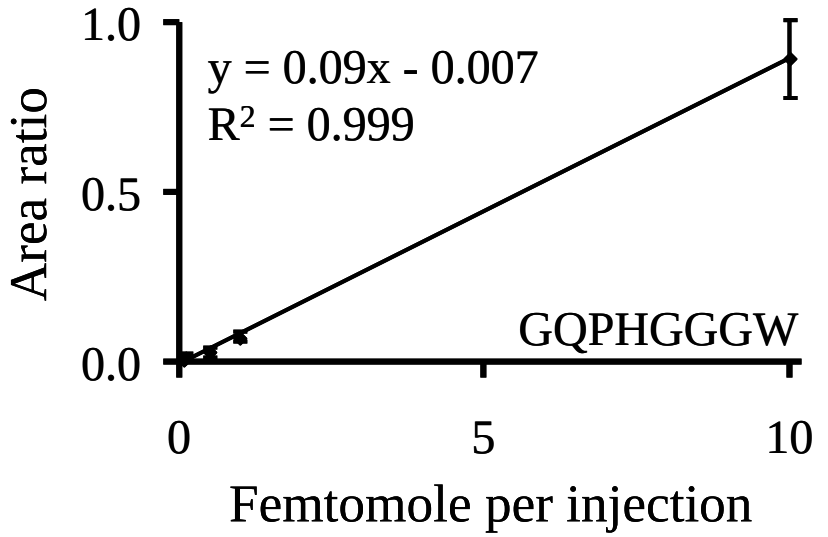


Figure S-8. Calibration curves for the sheep peptide, GQPHGGGW. This calibration curve was used to determine the empirical relationship between the concentration of the GQPHGGGW peptide and the R_{159}YPNQVY$_{165}$ peptide used as an internal standard. All concentrations are reported as the mean ± standard deviation of four injections.

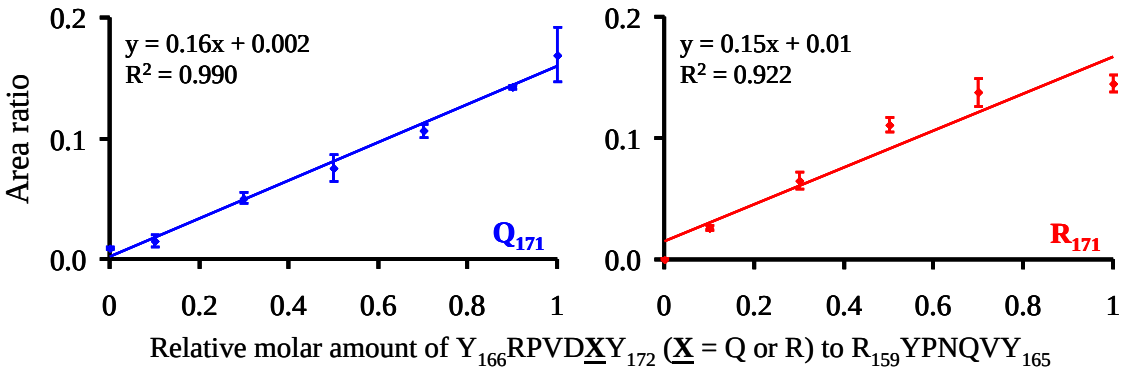


Figure S-9. Relationship between the area ratios of signals from varying concentrations of the peptides Y_{166}RPVD**Q**Y_{172} or Y_{166}RPVD**R**Y_{172} to a fixed concentration of the peptide R_{159}YPNQVY$_{165}$.

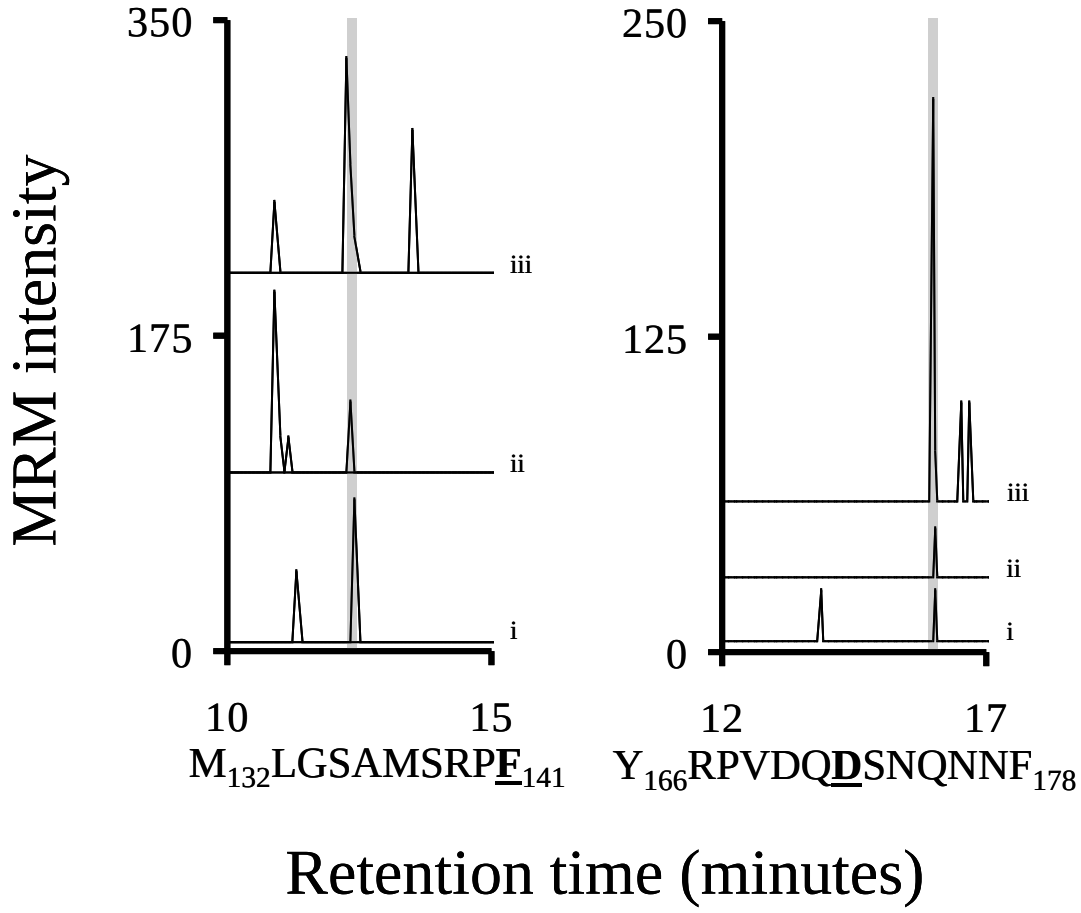
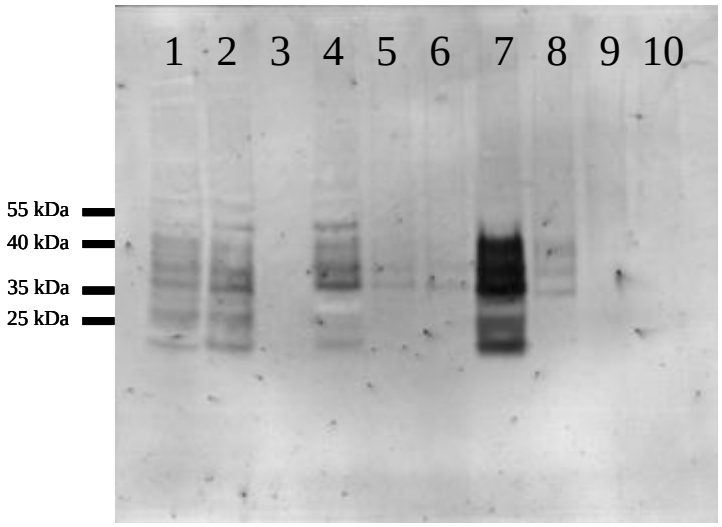


Figure S-10. Graphs showing the limit of detection (LOD) for the two chymotryptic peptides (M_{132}LGSAMSRP**F**$_{141}$ and Y_{166}RPVDQ**D**SNQNNF$_{178}$). The signal intensity of the optimal ion of each peptide from either a 50 (i), 100 (ii), or 500 (iii) attomole injection is shown and is offset for clarity. The grey shaded area delineates the expected retention time based on the added internal standard. The graphs show the relative retention times of the peptide.



Lane	Sample	Amount (fmol)
1	Flow Through	$7.1 \times 10^2 \pm 8 \times 10^1$
2	5% Sark/4M GuCl	$2.8 \times 10^2 \pm 2 \times 10^1$
3	4M GuCl	$6 \times 10^1 \pm 2 \times 10^1$
4	20 mM Imidazole/4M GuCl	$2.7 \times 10^2 \pm 8 \times 10^1$
5	50 mM Imidazole/4M GuCl	$6 \times 10^1 \pm 2 \times 10^1$
6	100 mM Imidazole/4M GuCl	7 ± 3
7	250 mM Imidazole/5% Sark/2M GuCl	$1.4 \times 10^3 \pm 7 \times 10^1$
8	250 mM Imidazole/5% Sark/2M GuCl	8 ± 5
9	100 mM EDTA/5% Sark/2M GuCl	9 ± 1
10	100 mM EDTA/ 5% Sark/2M GuCl	6 ± 1

Figure S-11. A Western blot-based monitoring of the isolation of PrP^C from a Bolton preparation supernatant. The starting sample was 100 mg of brain tissue and each lane corresponds to approximately 1.5 % of the sample. The primary mAb is GE8 and the secondary mAb is the goat anti-mouse Fc. The migration of the lane markers is shown on the left. The amount in each sample was determined by mass spectrometry.

References

(1) Onisko, B.; Dynin, I.; Requena, J. R.; Silva, C. J.; Erickson, M.; Carter, J. M. *J. Am. Soc. Mass Spectrom.* **2007**, *18*, 1070-1079.

(2) Boelaert, F.; Hugas, M.; Pelaez, A. O.; Rizzi, V.; Stella, P.; Van Der Stede, Y. *EFSA Journal* **2016**, *14*, 4643.

(3) Langeveld, J. P.; Jacobs, J. G.; Erkens, J. H.; Bossers, A.; van Zijderveld, F. G.; van Keulen, L. J. *BMC Vet Res* **2006**, *2*, 19.

(4) O'Rourke, K. I.; Baszler, T. V.; Miller, J. M.; Spraker, T. R.; Sadler-Riggleman, I.; Knowles, D. P. *J Clin Microbiol* **1998**, *36*, 1750-1755.

(5) Fediaevsky, A.; Maurella, C.; Noremark, M.; Ingravalle, F.; Thorgeirsdottir, S.; Orge, L.; Poizat, R.; Hautaniemi, M.; Liam, B.; Calavas, D.; Ru, G.; Hopp, P. *BMC Vet Res* **2010**, *6*, 9.

(6) Tveit, H.; Lund, C.; Olsen, C. M.; Ersdal, C.; Prydz, K.; Harbitz, I.; Tranulis, M. A. *Biochem Biophys Res Commun* **2005**, *337*, 232-240.

(7) Watt, N. T.; Taylor, D. R.; Gillott, A.; Thomas, D. A.; Perera, W. S.; Hooper, N. M. *J Biol Chem* **2005**, *280*, 35914-35921.

(8) Goldmann, W.; Hunter, N.; Foster, J. D.; Salbaum, J. M.; Beyreuther, K.; Hope, J. *Proc Natl Acad Sci U S A* **1990**, *87*, 2476-2480.

(9) Monleon, E.; Monzon, M.; Hortells, P.; Vargas, A.; Acin, C.; Badiola, J. J. *J Histochem Cytochem* **2004**, *52*, 145-151.

(10) Bolton, D. C.; Rudelli, R. D.; Currie, J. R.; Bendheim, P. E. *J. Gen. Virol.* **1991**, *72*, 2905-2913.

(11) Silva, C. J.; Onisko, B. C.; Dynin, I.; Erickson, M. L.; Requena, J. R.; Carter, J. M. *Anal. Chem.* **2011**, *83*, 1609-1615.

(12) Gretzschel, A.; Buschmann, A.; Langeveld, J.; Groschup, M. H. *J Gen Virol* **2006**, *87*, 3715-3722.

(13) Prusiner, S. B.; Groth, D.; Serban, A.; Stahl, N.; Gabizon, R. *Proc. Natl. Acad. Sci. U. S. A.* **1993**, *90*, 2793-2797.

(14) Barik, S. *Mol. Biotechnol.* **1995**, *3*, 1-7.

(15) Xu, Z.; Colosimo, A.; Gruenert, D. C. *Methods Mol. Biol.* **2003**, *235*, 203-207.

(16) U.S.-NIH *Biosafety in Microbioloical and Biomedical Laboratories, 5th Edition (https://www.cdc.gov/biosafety/publications/bmbl5/bmbl.pdf)*; Government Printing Office: Washington, D. C., 2009.