Prion protein quantification in human cerebrospinal fluid as a tool for prion disease drug development

Supplementary Materials

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Supplementary Discussion

Technical parameters of the BetaPrion® ELISA kit.

As noted in Table 1, for one sample included as an inter-plate control on 17 different plates, we observed an inter-plate CV of 22%. The 17 plates included in our analysis include plates from three different manufacturer lots, run by two different operators (SV and EVM), read on two different platereaders (Fluostar Optima and Spectramax), all of which factors may contribute to the variability we observed.

On an intra-plate basis, we also observed slightly higher variability when including dilutions than when only comparing replicates at a single dilution (CV=11% vs. 8%). Most samples were analyzed at two dilutions, 1:10 and 1:50, with two replicates each. In many cases, one dilution or the other fell outside the assay's dynamic range, but among *N*=87 samples for which both the 1:10 and 1:50 dilutions had both replicates fall within the dynamic range of the assay (1 to 20 ng/mL final), the PrP level indicated by the 1:10 dilution was on average 3.5% higher than the 1:50 dilution.

Plate position effects.

To assess whether plate position affects apparent PrP levels in ELISA, we ran two whole ELISA plates loaded with technical replicates of the same CSF sample (v1209 with 0.03% CHAPS). One plate was loaded with a single channel pipette taking 29 minutes (Figure S2A-B) and the other was loaded with a multichannel pipette taking 11 minutes (Figure S2C-D). A visually subtle, yet significant (*P* = 1.5e-14, linear regression), decline in apparent PrP level is seen across the plate. For instance, in Figure S2A, the ten replicates loaded last (wells G9-H6) are on average 22% lower than the ten replicates loaded first (wells A11-B8). Adjustment based on the standard curves abolishes this slope, and reduces the CV among technical replicates (Figure S2B and D).

Spike recovery experiments.

While we ultimately achieved 90.5% recovery of recombinant human PrP spiked into CSF, this successful outcome was preceded by a number of experiments that usefully illuminate constraints of working with both the BetaPrion® ELISA assay and CSF PrP as an analyte. In our first experiment, recombinant full-length human PrP with concentration orthogonally established by amino acid analysis (AAA) was spiked into two CSF samples previously established to have high and low baseline PrP. Compared to the expected recovery, the recombinant protein gave a much higher signal than expected, with 392-451%, over-recovery (Figure S3A). This surprising finding suggested to us that the concentration of PrP in kit standards may be lower in practice than the stated concentration. To test this hypothesis, we directly compared the kit standard curve to a matched standard curve prepared with our recombinant PrP. This experiment confirmed that kit standards appeared lower than AAA-quantified PrP standards by a factor of roughly 4 (Figure S3B). We conclude that kit standards, while technically reproducible, may most usefully inform relative rather than absolute quantification of PrP.

We next attempted to assess spike recovery in an internally consistent system by comparing recombinant PrP spiked into CSF to a recombinant PrP standard curve. We diluted recombinant PrP in CSF, then serially diluted into additional CSF to create a five-

point series. The series of samples was re-frozen and measured by ELISA the next day. Under these intensive handling conditions, we observed only ~50% recovery even though the samples contained 0.03% CHAPS (Figure S3C). We hypothesized that the CHAPS additive, while helpful, could not fully protect against the high levels of plastic exposure involved in serial dilution of CSF. To test this hypothesis, we redid the experiment in C with special attention to protecting PrP from plastic adsorption. Recombinant PrP was diluted in blocking buffer to prepare a series of solutions at 100x the desired final concentrations of points in the spike series. These samples were then added to CSF aliquots at a 1:100 concentration, and used in a same-day ELISA experiment. With this level of attention to plastic exposure and the elimination of an additional freeze-thaw cycle relative to the standard curve, PrP was preserved near expected levels with 90.5% recovery observed (Figure S3D).

Finally, to assess recovery from a different angle, we titrated a high-PrP CSF sample into a low-PrP CSF sample at varying ratios, again ensuring minimal and consistent CSF handling. Under these conditions, we observed linear and proportional recovery of PrP (Figure S3E). These experiments provide additional evidence that the quality of PrP measurement afforded by the BetaPrion® ELISA assay is dependent on appropriate sample processing.

CSF aliquot size and PrP loss.

We observed that when working with experimental aliquots of CSF, lower volume aliquots appeared to have consistently lower PrP levels (Figure S5A). This effect is likely due to increased exposure of the sample to plastic due to the higher surface area to volume ratio in the polypropylene storage tube. This explanation would be consistent with observed PrP loss across multiple regimens of plastic exposure (see Figure 2). Notably, while aliquot size profoundly impacts PrP recovery from small (< 100 µL) aliquots, it does not appear to impact PrP levels in substantially larger CSF volumes. When comparing 1, 3 and 5 mL draws of a pooled CSF sample into identical 5 mL syringes, we did not see a difference in measured PrP (Figure S5B). The cylindrical shape of the syringe could also contribute to this finding, as the surface-area-to-volume ratio difference between different syringe volumes is less dramatic than that for very small sub-aliquots. These data have clinical implications: while downstream sub-aliquotting and storage can impact PrP levels, different syringe volumes during LPs performed with gentle aspiration will not greatly influence PrP recovery.

Handling of test-retest samples.

We analyzed within-subject test-retest reliability of CSF PrP in four cohorts (Figure S7). Here is what we know about the handling history of these samples:

- Metformin trial placebo controls (Steven Arnold). Mean CV = 13% (Figure 3 and Figure S7A). *N*=18 samples comprise 2 lumbar punctures from each of 9 placebo-treated individuals from a randomized trial of metformin in individuals with mild cognitive impairment due to either Alzheimer disease or suspected non-amyloid pathology (SNAP). Test-retest interval ranged from 8 to 11 weeks. Lumbar punctures were performed fasting between 8:00a and 10:00a. CSF samples were handled according to a uniform protocol by the same staff, aliquotted into 0.5 mL aliquots within 1 hour of collection and then frozen on dry ice before storage at -80°C. The aliquots we received, approximately 1.75 years

after the last sample was collected, were all 0.25 mL, indicating another round of freeze/thaw and aliquotting had occurred in the interim, but all samples were received in identical tubes with identical labeling.

- Sapropterin dihydrychloride trial participants (Kathryn Swoboda). Mean CV = 33% (Figure S7B). *N*=28 samples comprise 3 lumbar punctures from 8 individuals and 2 lumbar punctures from 2 individuals, all with Segawa syndrome (biallelic *GCH1* loss-of-function), enrolled in a trial monitoring effects of sapropterin dihydrochloride on CSF biomarkers. Test-retest interval ranged from 5 to 25 weeks. Lumbar punctures were performed at various times of day. Details of sample handling history are not known, but the aliquots we received were of various sizes (range: 150 µL to 1.3 mL) and were stored in different types of tubes (screw cap and flip top) with varied labeling (electronically generated and hand-written), suggesting a diverse sample handling history.
- MIND external lumbar drains (MGH MIND Tissue Bank). Mean CV = 40% (Figure S7C). *N*=18 samples comprise 3 days of external lumbar drains from 4 patients and 2 days of lumbar drains from 3 patients, with a test-retest interval ranging from 1 day to 4 months. These individuals were being evaluated at MGH for normal pressure hydrocephalus (*N*=7), *C. dificile* infection (*N*=1), or *Herpes simplex* infection (*N*=1). CSFs from these in-patient lumbar drains had contact with diverse plastics for varying amounts of time before freezing. In general, the samples passed through a pressure-measuring burette made of cellulose acetate propionate (CAP) before draining into a polyvinyl chloride (PVC) bag. CSF was later collected from the bag and frozen in either polystyrene (PS) or polypropylene (PP) tubes. Aliquots we received were of two different sizes: 0.5 mL and 4.0 mL.
- Pre-symptomatic and symptomatic PRNP mutation carriers (Michael Geschwind). Mean CV=34% in each (Figure S7D-E). Samples were collected between 2009 and 2017 at two sites (UCSF Parnassus NIH GCRC/CTSI and subsequently on the UCSF Mission Bay Neuroscience Clinical Research Unit) with multiple different physicians performing lumbar punctures according to a uniform protocol. Test-retest interval ranged from 2 months to 6 years. Samples were collected at various times of day and kept under refrigeration for variable amounts of time, ranging from a few hours to overnight, before being sent to UCSF CoreLabs. Samples collected prior to September 2016 were frozen immediately upon receipt at CoreLabs, and were later thawed and aliquotted in the first half of 2017. Beginning September 2016 CoreLabs aliquotted the samples upon receipt using polypropylene pipette tips (Rainin RT-L1000F) into 0.5 mL cryovials (Fisher 02-681-333) prior to first freeze. The sub-aliquots that we received were in identical tubes with uniform labels, and were all labeled as being 250 µL, however, we found that the actual recoverable volume in each tube varied, with some as low as 100 µL; all data reported here are from aliquots with at least 140 µL.

Supplementary Table

Cohort (Collaborator)	*N*	Diagnosis	Description
Metformin trial (Steven Arnold)	18	Alzheimer disease and MCI-SNAP	Placebo-treated controls from a randomized trial monitoring effects of metformin on CSF biomarkers(33). 8-11 week test-retest. Samples were handled uniformly (see Supplementary Discussion) and were centrifuged prior to freezing.
MGH MIND Tissue Bank	27	NPH, *C. dificile*, herpes simplex	Large volume assay development samples from NPH patients (*N*=9), test-retest lumbar drains (*N*=18), and lumbar-thoracic gradient samples (*N*=8). Samples were centrifuged for 10 minutes at 2,000xG after receipt in our lab.
Sapropterin trial (Kathryn J Swoboda)	28	Segawa syndrome (*GCH1* loss of function)	Patients who received sapropterin dihydrochloride in a trial monitoring effects on CSF biomarkers (*N*=10 individuals). 5-25 week test-retest. Samples were centrifuged for 10 minutes at 2,000xG after receipt in our lab.
Bologna prion referrals (Piero Parchi)	34	Symptomatic prion and non-prion dementias	Dementia patients referred to the CJD Reference Center at University of Bologna due to suspected prion disease. Samples are autopsy-confirmed positive or negative for prion disease. Prion samples include sporadic and genetic (E200K, *N*=5). Prior to arriving at Dr. Parchi's lab from referring physicians, samples were variably centrifuged or not, and variably shipped frozen, cold, or at room temperature. Samples not marked as previously centrifuged were centrifuged for 10 minutes at 2,000xG after receipt in our lab.
Göttingen prion referrals (Inga Zerr)	29	Symptomatic prion and non-prion dementias	Dementia patients referred to the CJD Reference Center at University of Göttingen due to suspected prion disease. Samples are autopsy-confirmed positive or negative for prion disease. Prion samples include sporadic and genetic (D178N, *N*=2; E200K, *N*=2; V210I *N*=2). Samples were centrifuged for 10 minutes at 2,000xG after receipt in our lab. These samples were received after the data in Figure 1 were generated, so we added 0.03% CHAPS prior to sub-aliquotting and ELISA.
Cognitive impairment (Henrik Zetterberg)	20	Cognitive impairment	Patients with undiagnosed cognitive impairment and normal levels of CSF tau, phospho-tau, and amyloid beta. Samples were centrifuged for 10 minutes at 2,000xG after receipt in our lab.
UCSF (Michael Geschwind)	61	Symptomatic and pre-symptomatic genetic prion disease	Participants with *PRNP* mutations in the Early Diagnosis of Human Prion Disease study at UCSF(47). The cohort includes *N*=61 samples from *N*=40 distinct individuals (28 pre-symptomatic and 12 symptomatic), with 1 to 5

			samples per person collected at intervals ranging from 2 months to 6 years. Mutations represented include P102L (*N*=4 individuals), D178N (*N*=6), E200K (*N*=16), and ten other mutations (details omitted to protect patient privacy), including five with literature evidence for high penetrance and five without (see companion paper by Minikel et al). These samples were received after the data in Figure 1 were generated, so we added 0.03% CHAPS prior to sub-aliquotting and ELISA. Samples were never centrifuged.
TOTAL	**225**		

Table S1. CSF samples analyzed.

Abbreviations: normal pressure hydrocephalus (NPH); mild cognitive impairment with suspected non-amyloid pathology (MCI-SNAP).

Supplementary Figures

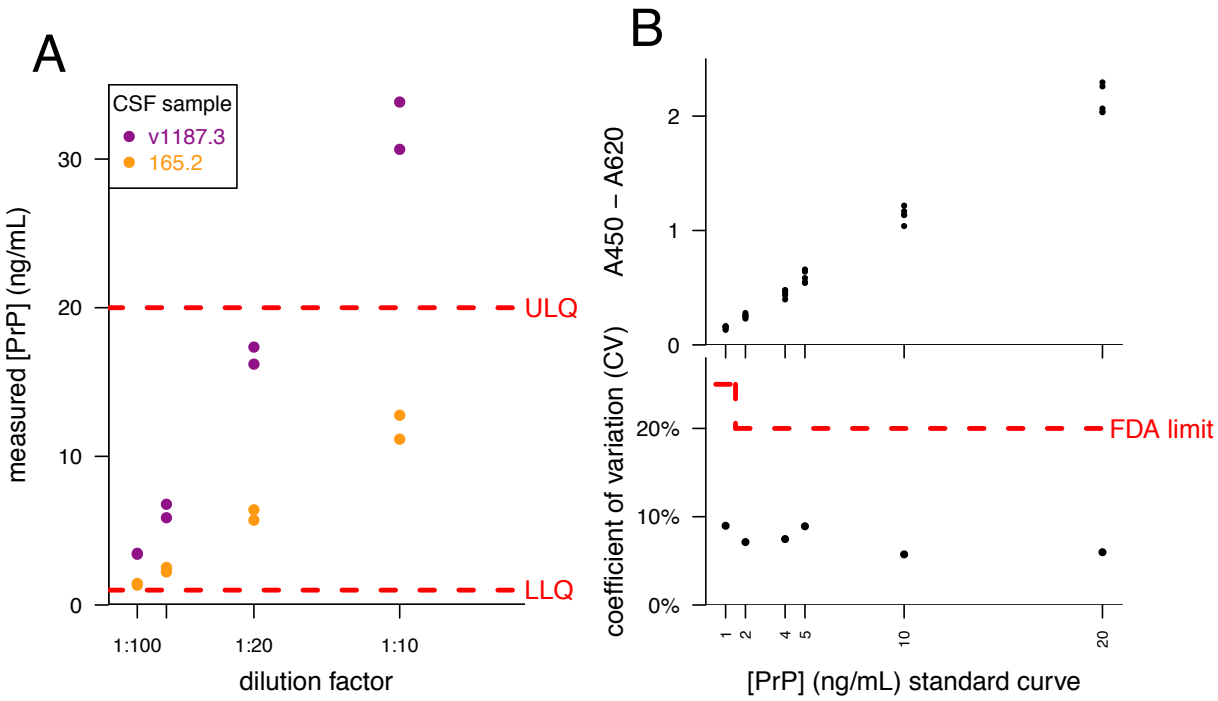


Figure S1. The BetaPrion® Human PrP ELISA kit quantifies PrP in a technically reproducible and sensitive manner.

A) Consistent dilution linearity was observed within the assay's stated dynamic range of 1 – 20 ng/mL PrP, providing reassurance that this technique can be used to compare PrP levels across samples even when these levels differ by one log. Purple and yellow dots represent two different samples measured in duplicate at each of four dilutions. B) Five replicates of the kit's internal six-point standard curve, reconstituted from lyophilized standards, were run in parallel on one plate. Across the dynamic range of the assay, the coefficient of variation falls below 10% for all points and well below the 20% FDA recommended limit in standard variability for ligand-binding assays.

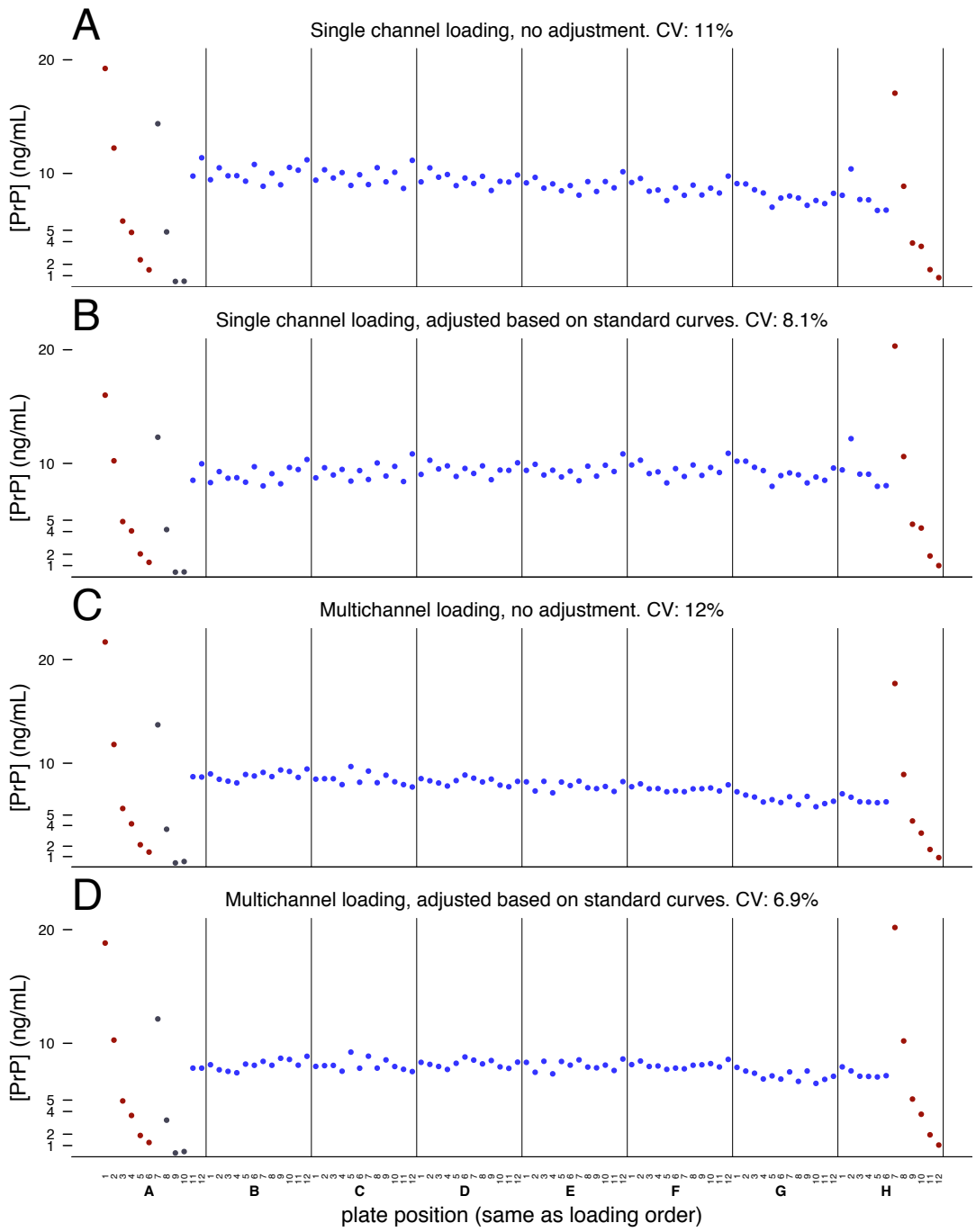


Figure S2. Plate position effects.

Computed PrP levels for standard curves (red), kit controls (gray), or the CSF sample (blue) in two whole plates loaded with technical replicates of the same CSF sample (NPH sample v1209 with 0.03% CHAPS) using either a single channel pipette (A-B) or a multichannel pipette (C-D). Displayed are the unadjusted PrP values (A and C) or the PrP values after adjustment based on the difference between the standard curves at the beginning and end of the plate (B and D). See supplementary discussion for further interpretation.

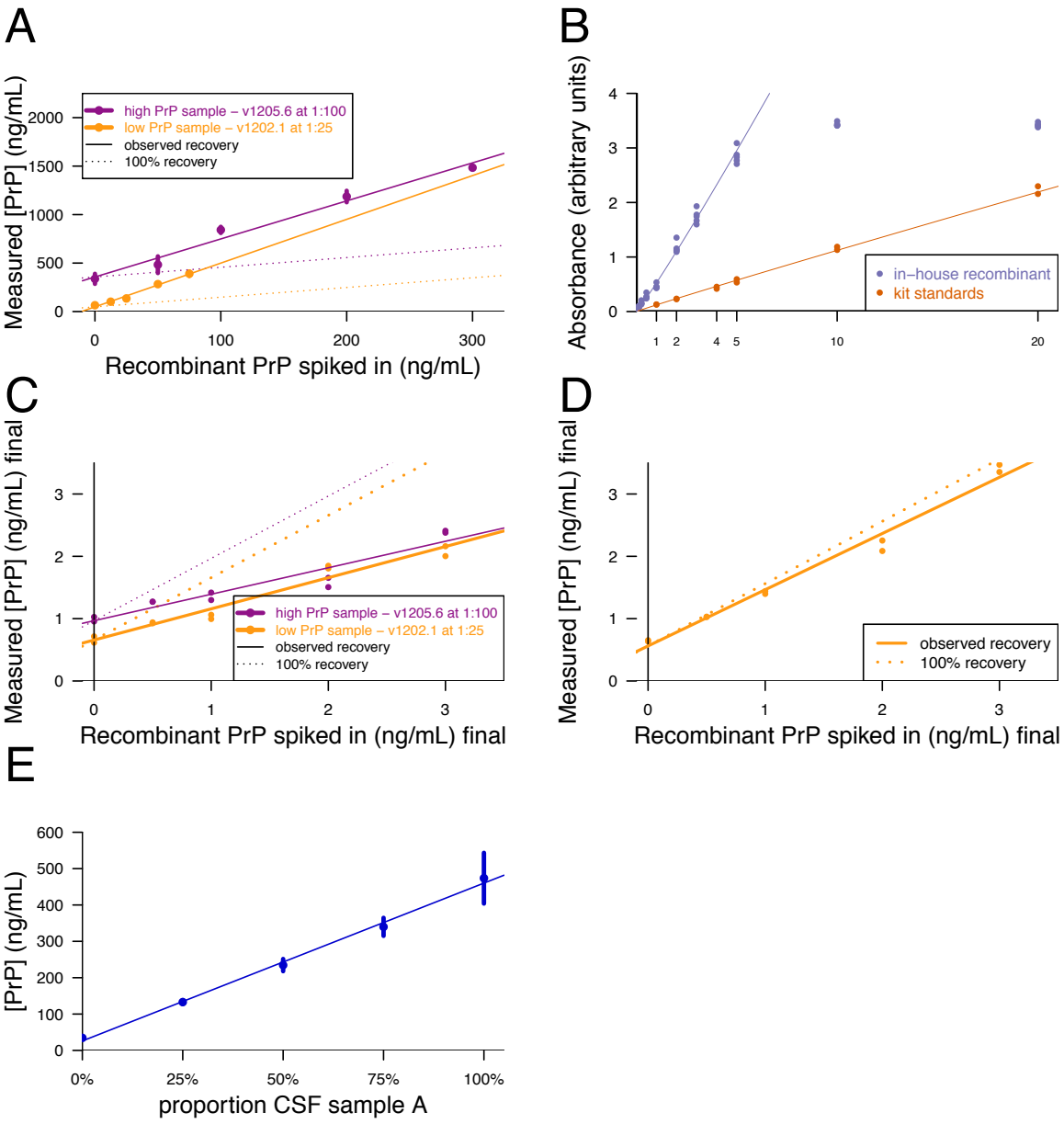
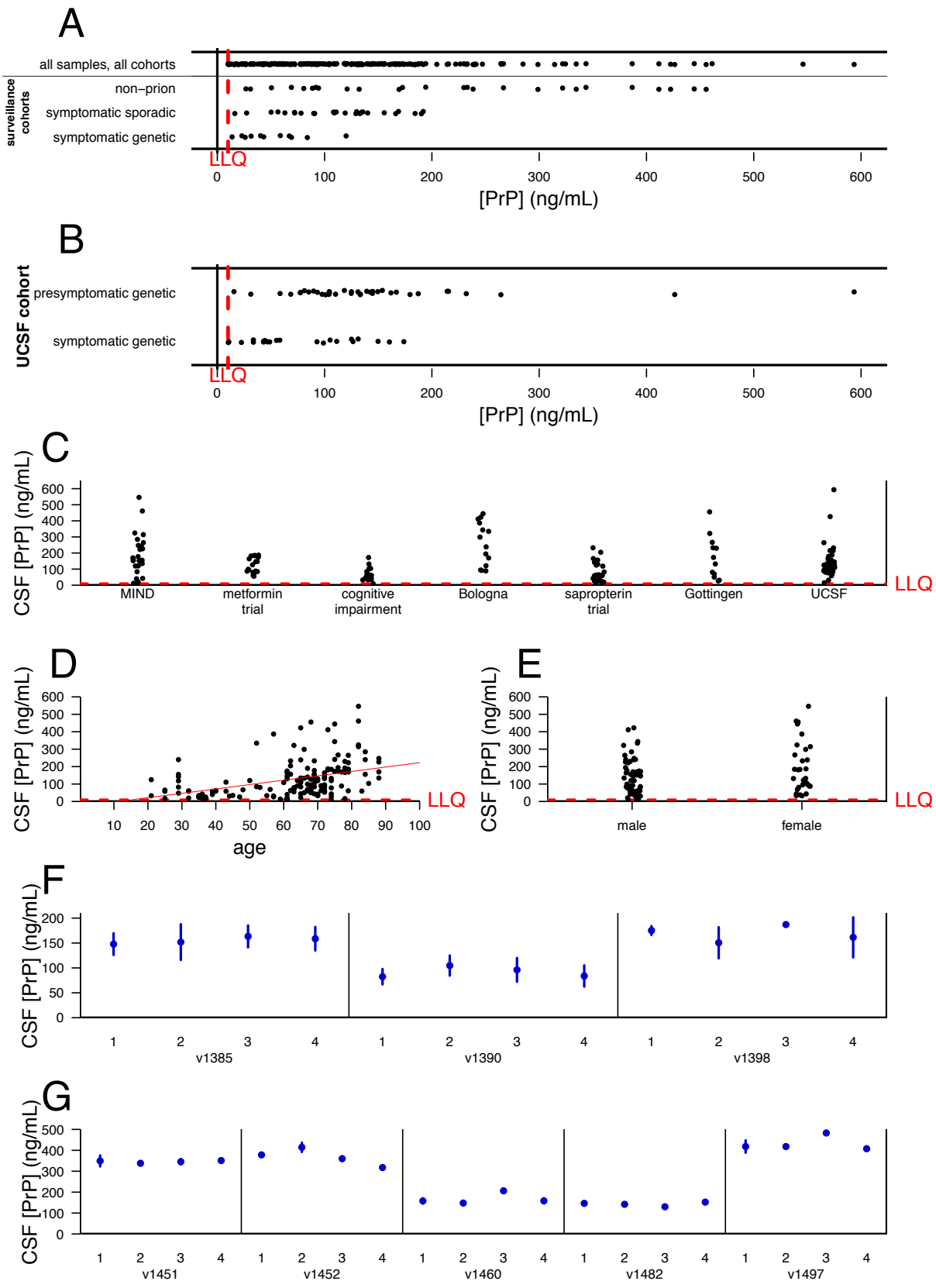


Figure S3. Spike recovery experiments.

A) In-house produced full-length recombinant human prion protein, quantified by amino acid analysis (AAA) was spiked into two CSF samples previously established to have high and low baseline PrP. Recombinant PrP was over-recovered by 392-451% (meaning that measured concentrations were ~4x the expected concentrations) when compared to kit standards. B) A recombinant standard curve was prepared from AAA-quantified recombinant huPrP to match the nominal concentrations of each of the six points on the BetaPrion® kit standard curve. Direct comparisons of the two series by ELISA showed the recombinant curve to be contain roughly 4x greater PrP at each point. C) Recombinant huPrP was measured according to a recombinant PrP standard curve. Recombinant PrP was diluted in CSF, then serially diluted into additional CSF to create a five-point series. The series of samples was re-frozen and measured by ELISA the next day. Under these conditions we observed 50.0% and 42.5% recovery for two different samples. D) The experiment in C was redone with the following modifications. Recombinant PrP was diluted directly in the initial aliquot tube with blocking buffer (5% BSA and 0.05% Tween-20 in

PBS, filtered prior to use). It was further diluted in blocking buffer to prepare a series of solutions at 100x the desired final concentrations of points in the spike series. These samples were then added to CSF aliquots at a 1:100 concentration. These samples were then diluted in blocking buffer to their final plating concentration and measured in a same-day ELISA experiment. Under these conditions we observed 90.2% recovery. E) A high-PrP CSF sample (sample A) was titrated into a low-PrP CSF sample at varying ratios, with minimal CSF handling. We observed linear recovery of PrP. See supplementary discussion for further interpretation.


A
all samples, all cohorts
surveillance cohorts
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symptomatic sporadic
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v1390
v1398
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CSF [PrP] (ng/mL)
v1451
v1452
v1460
v1482
v1497

Figure S4. Candidate explanations for variability in CSF PrP levels.

A) Within cohorts of individuals referred with a possible diagnosis of prion disease (Göttingen and Bologna cohorts), PrP levels are lower in individuals with prion disease than in individuals with other diagnoses. PrP levels in sporadic prion disease CSF average 42% of non-prion samples (P = 0.0001, Kolmogorov-Smirnov test) and in genetic prion disease CSF average 19% of non-prion samples (P = 2.6e-6, Kolmogorov-Smirnov test). B) Among individuals with a PRNP *mutation (UCSF cohort), PrP levels in symptomatic individuals average 53% of those in pre-symptomatic individuals (P = .001, Kolmogorov-Smirnov test). C) CSF PrP levels vary dramatically between different cohorts in our study, even after excluding individuals with symptomatic prion disease (P = 1.1e-8, Type I ANOVA). D) CSF PrP is positively correlated with age (r = 0.47, P = 1.9e-9, Spearman rank test), although among our samples age is confounded with cohort, diagnosis, and likely with other unobserved variables, so it is unclear whether this correlation is biologically meaningful. For example, consider symptomatic prion disease patients in the two prion surveillance cohorts (Bologna and Göttingen). Symptomatic genetic patients were on average younger than symptomatic sporadic patients (mean 55 vs. 68 years old,* P *= 0.001, Kolmogorov-Smirnov test), and controlling for genetic vs. sporadic diagnosis eliminated any trend towards correlation between age and CSF PrP (linear regression,* P *= 0.37 with diagnosis as covariate,* P *= 0.04 without). E) Excluding individuals with symptomatic prion disease, CSF PrP does not differ between men and women (P = 0.31, Kolmogorov-Smirnov test). F) CSF PrP exhibits no lumbar-thoracic gradient within ~30 mL intrathecal CSF drips. From each of three individuals with normal pressure hydrocephalus, 29-32 mL of intrathecal CSF was collected via drip in 4 polystyrene tubes of 7-8 mL each, with "1" being the first tube and "4" being the final tube. Because CSF from further up the spinal column is expected to drain downward as CSF is removed, "1" represents the most lumbar CSF while "4" is the most thoracic. PrP exhibits no trend across tubes (*P *= 0.81, linear regression). Error bars show technical replicates performed in duplicate. G) CSF PrP likewise exhibits no lumbar-thoracic gradient when ~20 mL of CSF is drawn using gentle aspiration with a 24G Sprotte needle. Approximately 5 mL of CSF was drawn in each of four syringes; again, "1" is the most lumbar and "4" is the most thoracic. These samples included individuals diagnosed with Alzheimer's disease, Parkinson's disease, and undiagnosed individuals. PrP exhibits no trend across syringes (*P *= 0.93, linear regression). Error bars show technical replicates performed in duplicate.*

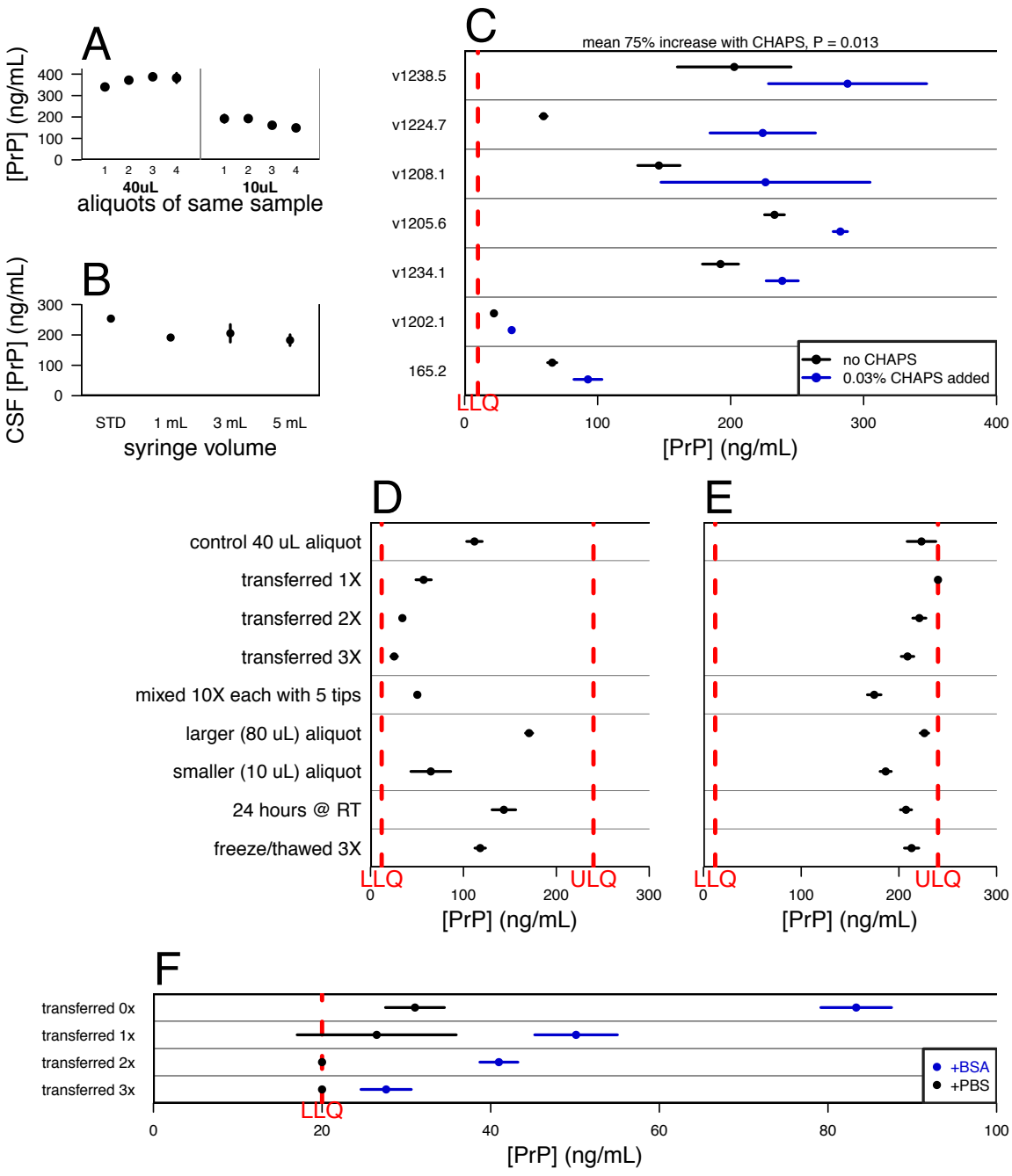

Figure S5. Additional evidence for loss of PrP to plastic adsorption.

A) Differently sized aliquots of sample v1187 appear to have different PrP levels. Each dot is the mean, and line segment the 95% CI, of two technical replicates on the same plate. These samples did not contain CHAPS. B) A pooled CSF standard (STD) was warmed to 37°C and various volumes (1 mL, 3 mL, or 5 mL) were drawn into identical 5 mL syringes using a 24G Sprotte needle and allowed to sit for 15 minutes before ejection into tubes, centrifugation, and aliquotting. Samples were handled identically except for the volume drawn into the syringe. See supplementary discussion. C) After aliquotting and freeze/thaw, CSF samples were diluted into blocking buffer neat (black) or after addition of a final concentration of 0.03% CHAPS to the

original storage tube (blue). Addition of CHAPS resulted in a 75% increase in apparent PrP level. See supplementary discussion. D and E) Replication of the findings from Figure 1A-B. The data in Figure 1 were generated using CSF samples from two different individuals; to rule out the possibility that some other inter-individual difference, rather than CHAPS, explained the difference in plastic loss, we repeated the experiment but with a single CSF sample divided into two halves which were then aliquotted without (D) or with (E) 0.03% CHAPS, subjected to the same battery of perturbations and plated at the same dilution. Because CHAPS increases overall PrP recovery, some replicates in (E) are at the upper limit of quantification; nevertheless, the results recapitulate Figure 1. F) 1 mg/mL (final concentration) BSA (blue), or PBS as a control (black), were added to CSF sample 165.2, which had an initial total protein level at the low end of the distribution of our samples (measured at 0.22 mg/mL with PBS), bringing it up to a total protein level at the high end of our samples (measured at 1.15 mg/mL after BSA spike-in). BSA or PBS were added after centrifugation but prior to aliquotting at 40 uL and re-freezing. 4 tubes of each sample were subsequently thawed and diluted into blocking buffer for analysis. Total recovery of PrP is increased in the BSA-spiked samples, analogous to panel B, although BSA is less effective at mitigating loss upon further transfer between tubes (compare to Figure 2A).

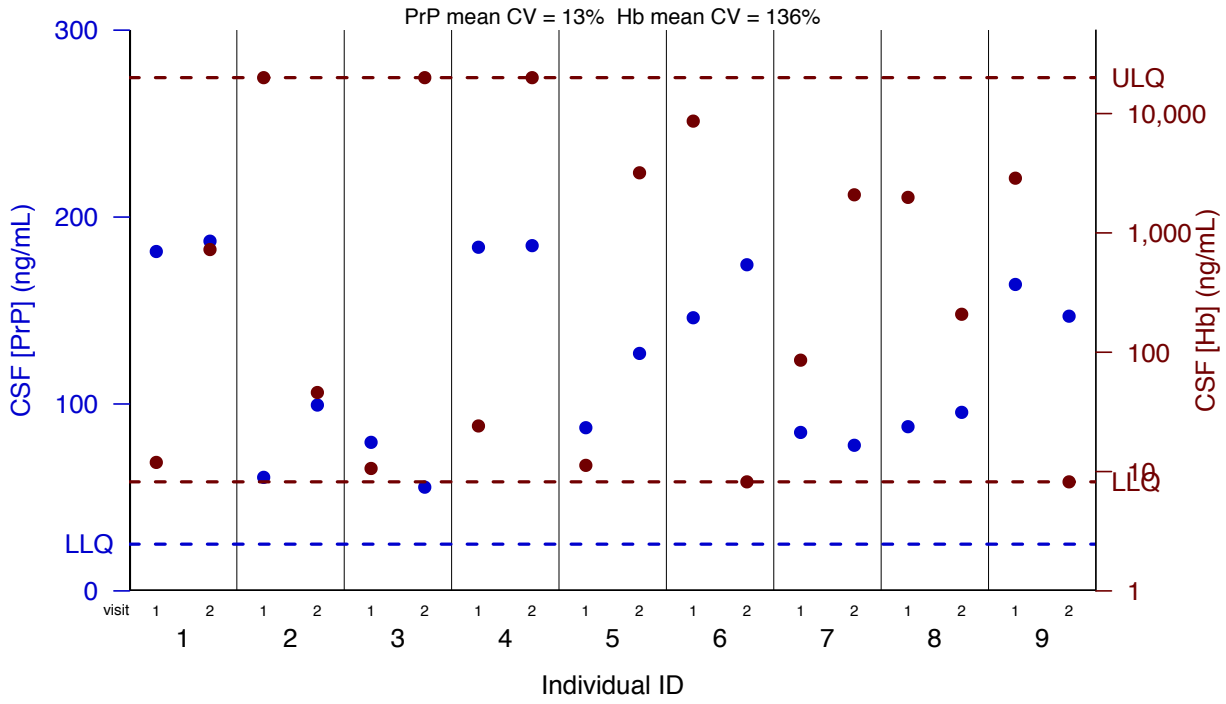

Figure S6. Hemoglobin in test-retest samples.

Overlaid are PrP levels (blue, same data as shown in Figure 3) and hemoglobin levels (red) in test-retest samples. PrP exhibited good test-retest reliability (mean CV=13%) despite dramatic variation in hemoglobin (mean CV=136%), providing further evidence that blood contamination does not influence CSF PrP level.

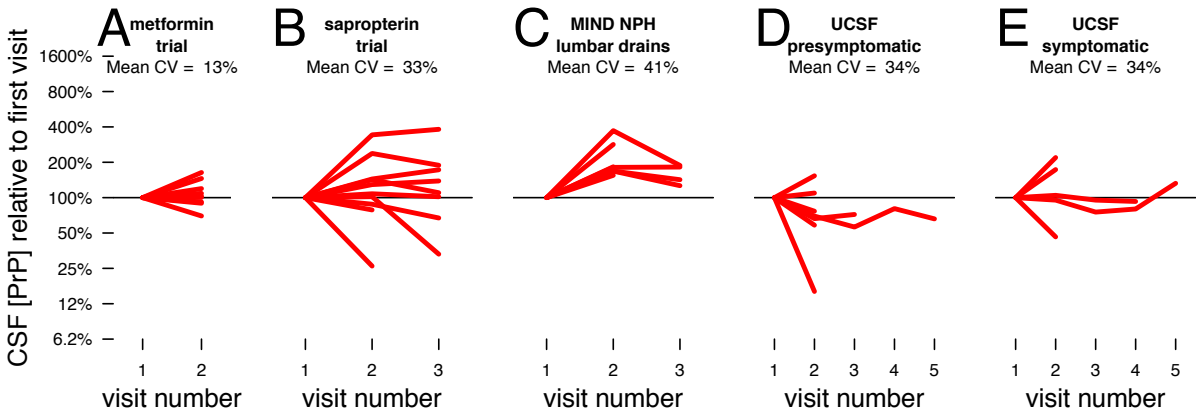

Figure S7. Test-retest reliability of CSF PrP in additional cohorts.

Test-retest CSF PrP levels in A) metformin trial participants (Arnold) over 8-11 weeks, with mean CV=13% (same data from Figure 3 but plotted normalized to the PrP level at the first visit); B) sapropterin dihydrochloride trial participants (Swoboda) over 5-25 weeks, with mean CV=33%, C) NPH lumbar drains (MGH MIND Tissue Bank) over 1 day to 4 months, with mean CV=40%, D) pre-symptomatic and E) symptomatic PRNP *mutation carriers (Geschwind) over 2 months to 6 years, each with mean CV=34%. The repeated 34% is not an error: the mean CVs in (D) and (E) happen to be the same (34.28% and 34.25%). See supplementary discussion for details on sample handling in these cohorts.*

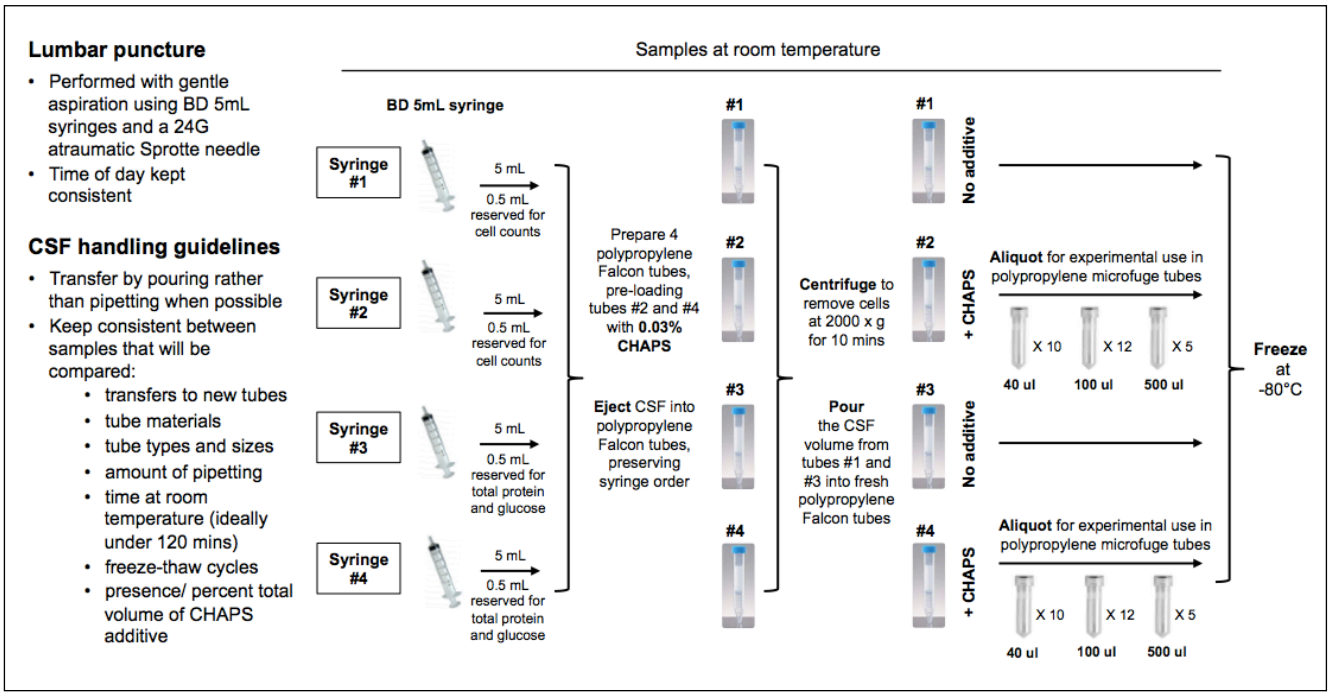

Figure S8. Protocol for collection of CSF for PrP measurement.

We have incorporated our findings into the above protocol, which we are using to collect test-retest CSF for the purposes of PrP measurement in our ongoing clinical study.