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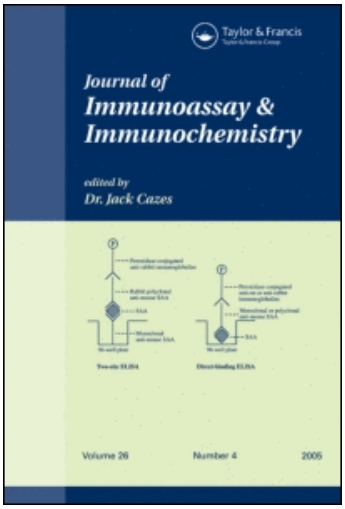

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Identification of Problems Developing an Ultrasensitive Immunoassay for the Ante Mortem Detection of the Infectious Isoform of the CWD-Associated Prion Protein

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Identification of Problems Developing an Ultrasensitive Immunoassay for the Ante Mortem Detection of the Infectious Isoform of the CWD-Associated Prion Protein

Benjamin Brooks,[1] Amanda Brooks,[1] Shaun S. Wulff,[2] and Randolph V. Lewis[1]
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Abstract: Ante-mortem assays exist for some Transmission Spongiform Encephalopathies (TSE). These assays facilitate our understanding of disease pathology and epidemiology; however, the limitations of these ante-mortem assays include the inability to quantify protein amount, poor sensitivity, and/or limited robustness. Here, we utilize a bioinformatics approach to report on problems associated with developing a more sensitive immunoassay for TSEs including: 1) the lack of specific and sufficiently sensitive antibodies for the infectious isoform(s) of PrP^{res}, 2) problems associated with serial titration of PrP^{res}, and 3) the distribution of PrP^{res} particle sizes. Overcoming these problems require more sophisticated antibody design and a creative engineering of an ultrasensitive protein assay systems for $PrP^{res.}$

Keywords: Bioinformatics study, Chronic wasting disease, Immunoassay, Prion antibodies, Prion protein, Ultrasensitive

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INTRODUCTION

Chronic wasting disease (CWD) is a naturally occurring transmissible spongiform encephalopathy (TSE) found in cervids primarily in North America.[1,2] TSEs in general and CWD specifically, are characterized primarily by the accumulation of misfolded prion protein (PrP) in central nervous system (CNS) tissues leading to death. The present ''gold standard'' diagnostic for TSEs is immunohistochemical screening (IHC) of brain tissues.[3] IHC are time and labor intensive currently taking from several days up to two weeks to complete. Western Blotting or other immunoassays are often utilized as these rapid tests take less time at 12–48 hours and are less labor intensive. However powerful these immunoassays are, all of these assays are limited in their sensitivity and rapidity; moreover, these assays are all limited to post mortem analysis.

A feature of CWD and scrapie is the primary infection of the lymphoreticular system (LRS). This fact allowed for the development of an ante mortem assay where lymph system tissues in CWD and the third eye lid in scrapie can accurately determine the infective status of the animal.[4,5] While this allows for ante mortem testing, these assays still suffer from limited sensitivity and rapidity with the additional problem of difficult sample acquisition. Presently, extensive research is being conducted in order to overcome these assay limitations with the hope that an assay can be developed that can sensitively and rapidly detect the misfolded prion protein in many fluids and tissues of a living animal shortly after infection.

Several new assays exist that either overcome some of these problems and/or provide a foundation for future ultrasensitive assays that potentially may address all of these problems. These new assays include 1) protein misfolding cyclic amplification (PMCA) technique,[6–11] 2) an *in vitro* fluorescent amplification catalyzed by T7 RNA polymerase assay (AS-FACTT),[12] and 3) conformation dependent immunoassay (CDI).[13–16] While these assays are a good start toward rapid, ultrasensitive detection of PrP^{res}, they continue to have a limited ability to quantify PrP^{res}, a limited ability to utilize different tissues or fluids, a limited portability, limited time efficiencies, and/or limited assay robustness. In addition the amplification assays have been susceptible to generating false positives with small changes in assay conditions.

Several generic ultrasensitive protein detection assay formats have been developed that exhibited potential for application in TSEs including antibody arrays, bio-bar code assays, and magnetic bead ELISAs. Extensive research was conducted in our labs utilizing these protein detection assay formats with little success. During these unsuccessful attempts we utilized several dozen antibodies in multiple assay formats and with

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different buffer conditions (data not shown). While unsuccessful at developing an immunoassay for detection of TSEs, attempts to apply these assay formats to other protein systems was successful and relatively straightforward. Discussion of why these assay formats failed in application to CWD tended to center on two general areas of concern including the problem of working with a distribution of infectious prion protein particle sizes and the problem of antibody sensitivity and specificity. Here, we utilize bioinformatics approaches to report on how standard immunoassay techniques, how the prion protein particle distribution, and how the lack of sufficient antibodies all combine to make the development of a sensitive and specific immunoassay difficult without further technological advances.

EXPERIMENTAL

Bioinformatics Analysis

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A prion particle size distribution was created from Silveira's work using flow field flow fractionation (FlFFF).[17] This particle distribution was an approximation of the number of prion particles in a brain sample for each given particle size. Three bioinformatics simulations were conducted based on this particle distribution. First, a C# random event algorithm was engineered to determine total protein amounts for serially titrated samples. We wanted to know how titrating prion protein affected the total amount of prion protein in each of the serially titrated samples.

Second, using a C++ random particle size selection algorithm based on the formula for the dissociation constant of the capture and target antibodies, simulations were performed using a perfectly titrated particle distribution. We assumed that a proportional amount of protein was in each titrated sample, i.e., a perfect titration. The only variability in the simulation was the variability due to the prion particle binding to an antibody as calculated by Equation 1.

$$K_D = \frac{[\text{Antibody}] \cdot [\text{Equivalent PrP}]}{[\text{Complex of Ab and PrP}]} \quad (1)$$

Because of the oligomeric nature of prion protein, each particle size has a different number of exposed epitopes. In order to simplify the analysis, we assumed only four conditions existed as diagrammed in Figure 2b. Because of the variation in exposed epitopes, Equation 2 was utilized in order to simulate the concentration of prion protein attached in an immunoassay sandwich.

$$[\text{Equivalent PrP}] = \frac{(\text{No. Exposed Epitopes per Particle} \times \text{Total No. of Particles})}{(\text{Avogadro's Number}/\text{Reaction Volume})} \quad (2)$$

In this experiment, we wanted to determine the limits of detection for a sandwich immunoassay for a given K_D of an antibody.

Finally, we inserted the titrated distribution from our first analysis into the algorithm utilized in our second analysis in order to determine overall characteristics of the immunoassay.

RESULTS AND DISCUSSIONS

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Infectious prion protein particles have a wide size distribution ranging from dimers to oligomers with greater than hundreds of units of individual prion protein monomers. A common practice in immunoassay development is the titration of a quantified sample in order to determine the dynamic range of the assay. Based on publication of a distribution of particle sizes for prion protein,[17] concern grew that a titration of the prion protein might cause wide variations in protein amount during a titration because of 1) particle distribution effects when titrating a PrP^{res} solution and 2) a failure of certain particles to be proportionally distributed when the total prion protein amount in a sample is less than a femtogram. A simulated probabilistic titration analysis was conducted, shown in Figure 1, and both effects described above appear to influence the amount of total prion protein in a sample during titration. The probabilistic effect of the particle distribution during a titration is suggested as the coefficient of variation (CV) of the total protein versus the predicted protein amount dramatically increases in all of the analyses as the number of times a sample is titrated increases. Further, as the titration reached the femtogram level, all of the analyses showed that the CV of the sample was approaching a level at which the total protein was a log off from predicted protein amounts. After conducting this analysis, an additional concern arose in that the variation in total signal might be amplified as a result in an ultrasensitive sandwich immunoassay format due to the number of exposed epitopes in prion particles.

Besides variation due to titration effects, a modeled immunoassay simulation was conducted in order to assess the potential variation associated with sandwich immunoassays (Figure 2). In particular, antibody sensitivity was a concern. Several areas of potential variation exist when conducting a computer modeled immunoassay including: the

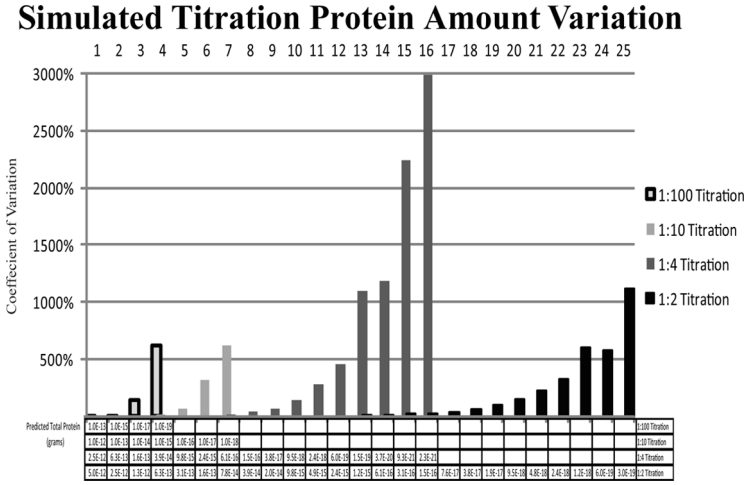

Figure 1. Simulation calculating variance of total protein amount when Titrating PrP^{res}. A computer simulation was conducted to determine the amount of variance of total protein while performing a standard titration of PrP^{res}. Initial distribution was calculated based on.[17,20] Three titrations were conducted: a 1:100 titration (1% of total volume removed), 1:10 titration (10% of total volume removed), and a 1:4 titration (25% of total volume removed). Predicted amount of total protein for each titration is show on the bottom of the graph.

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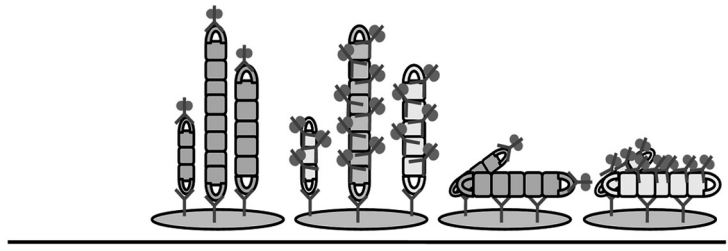
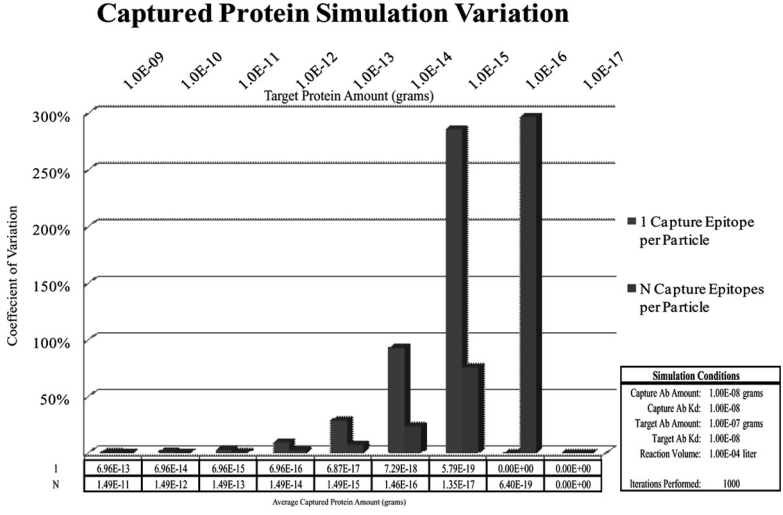
particle distribution, the amount of protein capture by the capture antibody, the target antibody affinity, the signal development process, the number of exposed epitopes, and titration effects as described earlier. In laboratory experiments additional variables include user variability and solution variability. In order to determine the variation due to the particle distribution and the number of exposed epitopes, a probabilistic immunoassay simulation was conducted on proportional distribution series calculated from standard size distribution (Figure 2). The standard size distribution assumes no variation due to titration. In these immunoassay simulations, the coefficient of variation (CV) was utilized as the measure. Algorithms utilized in the simulation calculated variation based on the particle distribution and number of exposed epitopes. The other potential areas of variability were not modeled in the simulation.

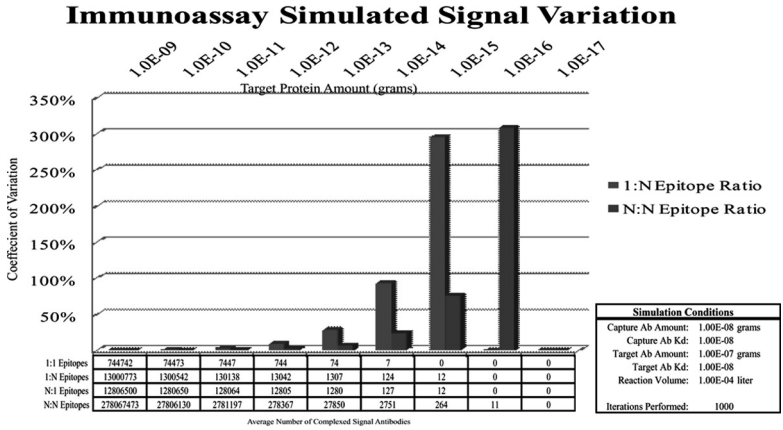
In addition to variability the sensitivity of the antibody was measured. This was done by assuming that the affinity of the antibody was determined by the number of exposed epitopes per particle. If a protein can be found in fibrils of different sizes, an antibody can have different affinities to the different particle sizes. In order to simulate



(a)



(b)



(c)

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the probabilistic effect of the distribution, two affinity states were utilized. In the first affinity state, there is only one epitope exposed per particle; whereas in the second, there are N epitopes exposed per particle where N is the number of monomers in a PrP fibril, i.e., if the oligomer was a pentamer, the number of exposed epitopes or N is five. Figure 2a shows the two states as applied to a sandwich immunoassay composed of a capture and target antibody. The number of exposed epitopes obviously affects the affinity of the antibody, and as well as the sensitivity as shown in Figures 2b and c.

The results of simulated sandwich immunoassay with a perfect serially titrated distribution (shown in Figures 2b and c) indicate that as the amount of total protein in an immunoassay approaches the desired sensitivity (fg/mL), the coefficient of variation (CV) rises to 10% at the fg/mL level and increases to over 200% at the ag/mL level. The effective simulated sensitivity of the immunoassay thus is in the range of fg/mL. The total capture protein and the total signal are utilized to determine the total variability of an assay. Total capture protein is the amount of protein captured by the capture antibody; whereas, total signal is the number of target antibodies attached. The relationship between total captured protein and total signal determines the variability in the simulation. Three additional facts are evident from the simulations performed in Figure 2. First, as the number of particles in the sample decreases, the coefficient of variation increases. This effect is due to a small sampling size as well as a breakdown in the distribution of particles as the amount of protein is reduced in each sample. Second as expected, the affinity of

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Figure 2. Captured protein and signal variation in computer simulated immunoassays with a standard distribution of PrP^{res} particles. (a) Graphic illustrating the number of exposed epitopes per particle. For example, if the exposed epitope is N and the particle size is seven, seven epitopes will be exposed per particle. The first number in the exposed epitope ratio is the capture (antibody attached to functionalized surface e.g. magnetic bead) and the second is the target antibody. If the ratio is N:1, then the capture antibody has N exposed epitopes per particle and the target has one. (b) The variance in the amount of protein captured by the capture antibody was measured in a simulation with a standard distribution (i.e., no variance assumed due to titration effects). The chart at the bottom of the graph represents the average number of particles captured by the capture antibody with the number of exposed epitopes per particle being either one or N. (c) The variance in the amount of signal, in this case the number of target antibody complexed in a sandwich with the prion particle and with the capture antibody, was measured in a simulation with a standard distribution. The chart at the bottom of the graph represents the average number of target antibodies complexed in the immunosandwich.

the antibody and consequently, the sensitivity of sandwich immunoassays are dependent on the number of epitopes exposed. As the number of epitopes per particle increase, the signal and thus the sensitivity of the immunoassay increases. One effect that is important is the increase in sensitivity and consequently, the variation that is experienced when more epitopes are exposed in the target antibody. Lastly, the K_D of the antibody is critical to both sensitivity and variation; however, even with increases in sensitivity due to a lower K_D, the increases in variation cause the resulting gains in sensitivity to decrease.

In order to assess the variation of both the titration and the variation of the sandwich immunoassay together, immunoassay simulations were conducted utilizing the titrated distributions shown in Figure 1. These experiments were conducted in order to determine the variability of a complete system. In these simulations shown in Figure 3, the amount of total protein in the sample, the amount of captured protein in a sample, and the amount of total signal are utilized to determine the variability of the sample. The average amount of total protein, capture protein and total signal as well as each variable's coefficient of variation are thus critical and are shown in Figure 3 in table form underneath the graph showing the CV. Again small CVs are desirable in these simulations, but more important in these simulations is the amount of the total signal. Samples where the total signal is greater than 40 particles and a CV of less than 10% are shown in green. Samples where total signal is greater than 40 (the limit of detection for antibody arrays) and the CV is between 10% and 100% are shown in yellow. These highlight conditions that are important as they represent the conditions where an immunoassay would be effective. Although the results show that the CV for a titrated distribution is worse, they are not as dramatically worse as one might expect. It is important to note that the variability in these simulations is limited as they do not include other variables commonly seen in the lab such as the assay format variation which could include protein capture variability, target Ab variability, and signal enhancement variability. The primary cause of variation in these simulations is due to particle size variation, and the secondary cause is the effects of titration. All three of the important facts outlined from Figure 2 also hold in these simulations.

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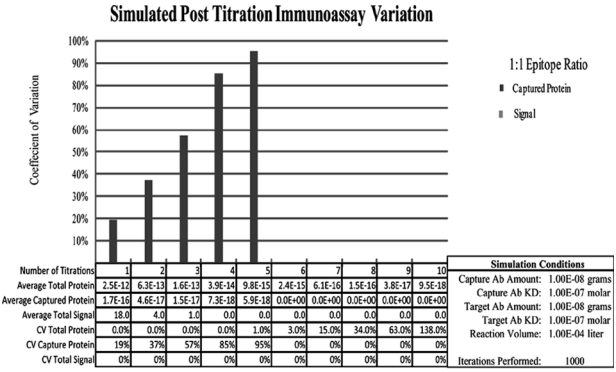
DISCUSSION

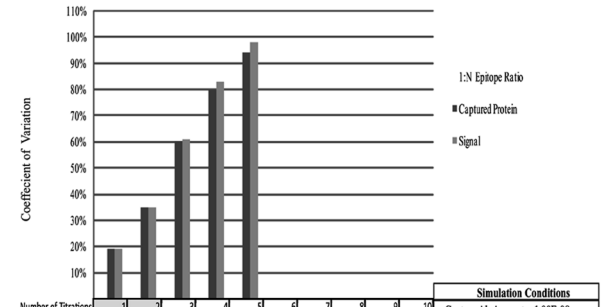
The driving force in PrP^{res} assay development has been specificity. This is due to several factors including political forces that focus on 100% specificity in order to minimize false positives, primary amino acid homology between conformations of prion protein, and poor antibody affinities and



	1	2	3	4	5	6	7	8	9	10
Number of Titrations	1	2	3	4	5	6	7	8	9	10
Average Total Protein	2.5E-12	6.3E-13	1.6E-13	3.9E-14	9.8E-15	2.4E-15	6.1E-16	1.5E-16	3.8E-17	9.5E-18
Average Captured Protein	1.7E-16	4.6E-17	1.5E-17	7.3E-18	5.9E-18	0.0E+00	0.0E+00	0.0E+00	0.0E+00	0.0E+00
Average Total Signal	18.0	4.0	1.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
CV Total Protein	0.0%	0.0%	0.0%	0.0%	1.0%	3.0%	15.0%	34.0%	63.0%	138.0%
CV Capture Protein	19%	37%	57%	85%	95%	0%	0%	0%	0%	0%
CV Total Signal	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%

Simulation Conditions	
Capture Ab Amount:	1.00E-08 grams
Capture Ab KD:	1.00E-07 molar
Target Ab Amount:	1.00E-08 grams
Target Ab KD:	1.00E-07 molar
Reaction Volume:	1.00E-04 liter
Iterations Performed:	1000

(a)



	1	2	3	4	5	6	7	8	9	10
Number of Titrations	1	2	3	4	5	6	7	8	9	10
Average Total Protein	2.5E-12	6.3E-13	1.6E-13	3.9E-14	9.8E-15	2.4E-15	6.1E-16	1.5E-16	3.8E-17	9.5E-18
Average Captured Protein	1.7E-16	4.5E-17	1.5E-17	7.7E-18	5.8E-18	0.0E+00	0.0E+00	0.0E+00	0.0E+00	0.0E+00
Average Total Signal	314.4	84.0	27.8	13.9	10.5	0.0	0.0	0.0	0.0	0.0
CV Total Protein	0.0%	0.0%	0.0%	0.0%	1.0%	3.0%	15.0%	34.0%	63.0%	138.0%
CV Capture Protein	19%	35%	60%	80%	94%	0%	0%	0%	0%	0%
CV Total Signal	19%	35%	61%	83%	98%	0%	0%	0%	0%	0%

Simulation Conditions	
Capture Ab Amount:	1.00E-08 grams
Capture Ab KD:	1.00E-07 molar
Target Ab Amount:	1.00E-08 grams
Target Ab KD:	1.00E-07 molar
Reaction Volume:	1.00E-04 liter
Iterations Performed:	1000

(b)

Figure 3. Captured protein and signal variation in computer simulated immunoassays with a titrated distribution of PrP^{res} particles. Simulated sandwich immunoassays were performed with titrated distribution from Figure 1. In the graph, the CV for the capture protein and the CV for the signal are shown for each group of immunoassays run against the titrated samples (series are not comparative against each other). Data from each simulation is shown below the titration number. Highlighted rows indicate that the simulation produced a total signal (i.e., the number of target antibodies attached) of greater than 40 and all CVs less than 100% but larger than 10%. (a) In this simulation, the epitope ratio was one exposed epitope per particle for the capture antibody and one exposed epitope per particle for the target antibody. (b) In this simulation, the epitope ratio was one exposed epitope per particle for the capture antibody and N exposed epitopes per particle for the target antibody. (c) In this simulation, the epitope ratio was N exposed epitope per particle for the capture antibody and one exposed epitopes per particle for the target antibody. (d) In this simulation, the epitope ratio was N exposed epitopes per particle for the capture antibody and N exposed epitopes per particle for the target antibody.

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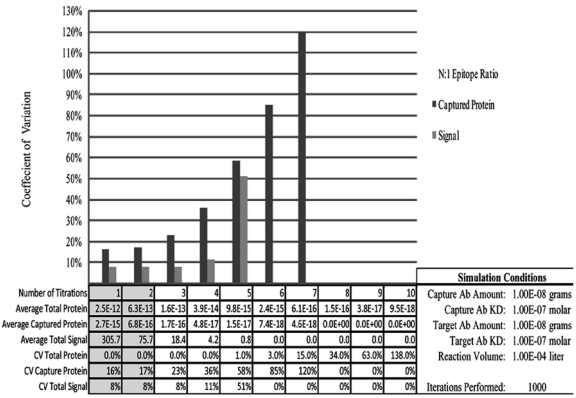
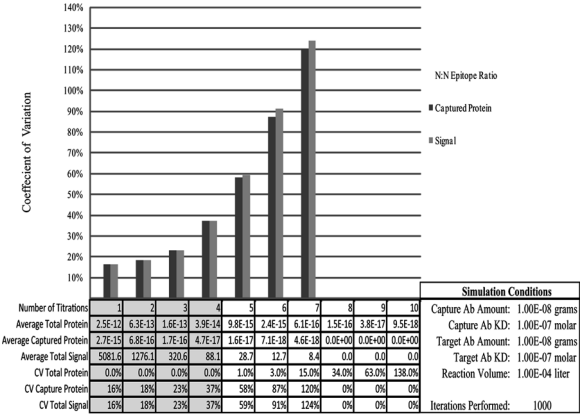


Number of Titrations	1	2	3	4	5	6	7	8	9	10
Average Total Protein	2.5E-12	6.3E-13	1.6E-13	3.9E-14	9.8E-15	2.4E-15	6.1E-16	1.5E-16	3.8E-17	9.5E-18
Average Captured Protein	2.7E-15	6.8E-16	1.7E-16	4.8E-17	1.5E-17	7.4E-18	4.6E-18	0.0E+00	0.0E+00	0.0E+00
Average Total Signal	305.7	75.7	18.4	4.2	0.8	0.0	0.0	0.0	0.0	0.0
CV Total Protein	0.0%	0.0%	0.0%	0.0%	1.0%	3.0%	15.0%	34.0%	63.0%	138.0%
CV Capture Protein	16%	17%	23%	36%	58%	85%	120%	0%	0%	0%
CV Total Signal	8%	8%	8%	11%	51%	0%	0%	0%	0%	0%

Simulation Conditions	
Capture Ab Amount:	1.00E-08 grams
Capture Ab KD:	1.00E-07 molar
Target Ab Amount:	1.00E-08 grams
Target Ab KD:	1.00E-07 molar
Reaction Volume:	1.00E-04 liter
Iterations Performed:	1000

(c)



Number of Titrations	1	2	3	4	5	6	7	8	9	10
Average Total Protein	2.5E-12	6.3E-13	1.6E-13	3.9E-14	9.8E-15	2.4E-15	6.1E-16	1.5E-16	3.8E-17	9.5E-18
Average Captured Protein	2.7E-15	6.8E-16	1.7E-16	4.7E-17	1.6E-17	7.1E-18	4.6E-18	0.0E+00	0.0E+00	0.0E+00
Average Total Signal	5081.6	1276.1	320.6	88.1	28.7	12.7	8.4	0.0	0.0	0.0
CV Total Protein	0.0%	0.0%	0.0%	0.0%	1.0%	3.0%	15.0%	34.0%	63.0%	138.0%
CV Capture Protein	16%	18%	23%	37%	58%	87%	120%	0%	0%	0%
CV Total Signal	16%	18%	23%	37%	59%	91%	124%	0%	0%	0%

Simulation Conditions	
Capture Ab Amount:	1.00E-08 grams
Capture Ab KD:	1.00E-07 molar
Target Ab Amount:	1.00E-08 grams
Target Ab KD:	1.00E-07 molar
Reaction Volume:	1.00E-04 liter
Iterations Performed:	1000

(d)

Figure 3. Continued.

specificities. Although specificity is critical to the development of an assay, research in sensitivity is also critical as it will open understanding of disease pathology and transmission. We have been dramatically less successful in developing a sensitive (<ng/mL), much less an ultrasensitive assay (<fg/mL). There are two main reasons for this lack of success. First, the antibodies developed to detect prion protein have poor affinities in sandwich immunoassays; moreover, they lose even more sensitivity in order to achieve specificity as samples require protease digestion, protein precipitation, or extensive processing in order to distinguish PrP^{res} from the normal conformation of prion protein. Based on simulations presented here, current antibodies have affinities in sandwich immunoassays of around 100 nM with little to no specificity in either

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ultrasensitive format and/or in body tissues and fluids, especially in samples with high levels of PrP^C.[19] While certain antibodies exhibit great specificity and sensitivity in non-sensitive assay formats like immunoblots, these same antibodies do not perform outside of these formats or without highly specific buffer conditions. It may be possible to engineer these antibodies to overcome these limitations both from an assay format perspective as well as a buffer perspective. Undoubtedly, antibodies are continually being generated for prion protein; however, antibodies need to be engineered for specific assay formats, for PrP^{res} to minimize sample processing, and for detecting multiple epitopes per particle possibly even for prion protein's posttranslational modifications. Lastly, prion protein's particle distribution also affects the sensitivity of these assays, in particular the limit of detection of the assay; however, the variation of the assay, as expressed in the CV, is the greatest casualty of the particle distribution.

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Infectious prion protein is found in a distribution of particle sizes ranging from pentamers to high order oligomers.[17,18] This distribution of particle size causes several unique properties related to variation in detection as shown in simulations described here. First, as the antibody reaches the limit of its K_D, the capture antibody sampling fails to follow the particle distribution due to the minimal captured sample size. The consequence is a dramatic increase in the variation in the amount of protein captured and thus a dramatic increase in the variation in assay signal (Figures 2 and 3). Second, another larger variation is due to the capture sampling effect which is seen when the amount of the total protein approaches attogram levels. The particle distribution in this situation fails leading to high variance in total signal. Third, the greater the number of exposed epitopes per particle leads to not only greater variance but also a corresponding loss in sensitivity. At some point, depending on the number of epitopes exposed per particle, an antibody engineered with high affinity will have decreasing sensitivity (i.e., non linear) due to a greater variability caused by particle distribution and the number of exposed epitopes. Lastly, if the number of titrations is large or if a small percentage volume titration is used, the variation increases almost logarithmically as the number of titrations is performed. This effect is likely to be seen in very dilute samples as well; however, this effect may not hold in other tissues and fluids as the particle distribution may be dramatically different than utilized in these experiments. This differential distribution is especially likely in fluids like urine where the kidney's filter is likely to filter larger particles more selectively. Nevertheless, several of these variations affect the amount of signal and will affect immunoassays as well as replicated assays such as QuIC and PMCA. These effects can combine resulting in huge variations of more than 1,000% leading to the erroneous identification of

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Table 1. Comparison of immunoassays

	Sensitivity	Specificity	Robustness	Quantitative	Tissue/Fluids	Rapid	Peer accepted
ELISA	++	++	+++	+++++	+++	+++++	+++++
Western	+	++++	++++	+++	+++++	++	+++++
IP	+	+++++	++++	++	++++	+	+++++
MB ELISA	++++	++	++++	++++	+++	+++	++++
BBCA	+++++	++	+	+++	+++	+++	++
Ab Array	++++	++	++	+++	+++	+++++	+++
PMCA	++++/+++++	+++	++/+++	+	+++	+	++
QuIC	++++/+++++	+++	++/+++	+	+	++	+
CDI	+++	++	++++	++	+++	++	+++
Ratings criteria							
+++++	<fg/ml	3 Unique Mechanisms	Highly Reproducible/ No PK	Small CV, Small Replicates	Non Tissue/Fluid Dependent	<2 hr Time	Clinically Accepted
++++	<500 fg/ml	2 Unique Mechanisms	Reproducible or PK Compatible	Either Small CV or Small Reps	Minimal Processing	<4 hr Time	Extensively Published
+++	<pg/ml	2 Unique/<2 Confounding	Reproducible but PK Problematic	Mod CV, Mod Reps	Buffer Dependent	<12 hr Time	Widely Published
++	<500 pg/ml	2 Dependent Mechanism	Reproducible in Volume	Either Mod CV, Mod Reps	Moderate Processing	<24 hr Time	Recently Published
+	<ng	1 Mechanisms	Poor Reproducibility	High CV, High Reps	Requires Extensive Processing	>24 hr Time	Unpublished

samples and false negatives; both situations are unacceptable. Solutions to these problems need to be addressed in order to develop an ultrasensitive assay.

The particle distribution also has one other very important effect. In amplification based assays such as QuIC (quaking induced conversion assay)[20] and PMCA, particle distribution can result in a false negative at low amounts of the protein. Based on titration simulations shown in Figure 1, this could be due to breakdown in particle distribution. Based on the results from Figure 1, it was hypothesized that because of the breakdown of the particle distribution (i.e., certain important particle sizes are not present at low protein amounts), a percentage of the assays would indicate in a negative result, although they were positive.

An ultrasensitive assay for PrP^{res} will need to meet the following characteristics: 1) highly specific for PrP^{res} in many tissues and fluid types even when PrP^{C} is present in high concentration, 2) highly sensitive (i.e., <hundreds of femtogram per mL), 3) quantitative with a 10% CV, 4) reasonably rapid (i.e., within two hours), 5) robust and reproducible, and 6) ante mortem. Current diagnostic methods for PrP^{res} have extremely limited sensitivity, portability, noninvasiveness, a high rate of false positives, and poor time efficiencies, although these limitations are getting better.[3] The most important aspect of the new assay will require sensitivity in the hundreds of femtograms per milliliter or better. Table 1 outlines the current available assay formats and an assessment of how the assay format meets the requirements outlined above. Presently, no assay format meets all of the requirements, although several formats have made significant progress recently. With additional fundamental research into these formats, an assay may lead us into critical areas of research such as characterization of transmission mechanisms and of disease pathology.

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