Design, implementation, and interpretation of amplification studies for prion detection

Nicholas J. Haley, Jürgen A. Rich, Kristen A. Davenport, Davin M. Henderson, Edward A. Hoover, Matteo Manca, Byron Caughhey, Douglas Marthaler, Jason Bartz, and Sabine Gilch

ABSTRACT
Amplification assays for transmissible spongiform encephalopathies have been in development for close to 15 years, with critical implications for the postmortem and antemortem diagnosis of human and animal prion diseases. Little has been published regarding the structured development, implementation, and interpretation of experiments making use of protein misfolding cyclic amplification (PMCA) and real-time quaking-induced conversion (RT-QuIC), and our goal with this Perspectives manuscript is to offer a framework which might allow for more efficient expansion of pilot studies into diagnostic trials in both human and animal subjects. This framework is made up of approaches common to diagnostic medicine, including a thorough understanding of analytical and diagnostic sensitivity and specificity, an a priori development of amplification strategy, and an effective experimental design. It is our hope that a structured framework for prion amplification assays will benefit not only experiments seeking to sensitively detect naturally-occurring cases of prion diseases and describe the pathogenesis of TSEs, but ultimately assist with future endeavors seeking to use these methods more broadly for other protein misfolding disorders, including Alzheimer’s and Parkinson’s disease.

Introduction
In 1985, a technique describing the amplification of short chains of DNA in vitro was first reported in Science – a technique which would soon revolutionize diagnostic testing in humans, animals, plants, and the environment [1-5]. In a few short years, the polymerase chain reaction (PCR) assay was adapted for use in diagnosing both genetic diseases and infectious agents, including what was arguably the most important and frightening pathogen of a generation – the human immunodeficiency virus [6].

Although PCR proved incredibly useful for detecting conventional pathogens, it was powerless for identifying a pathogen in the absence of nucleic acids, or one lacking nucleic acids altogether. Less than two decades later, an assay which made use of a similar strategy – the templated amplification of a target agent, a pathogen which could at once be spontaneous or genetic, transmissible, or both – was developed for a group of protein-only diseases which synchronistically created public fear: the prion diseases, or transmissible spongiform encephalopathies (TSEs) [7]. In vivo, the infectious, misfolded prion (PrPSc or PrPd) replicates by coercing normally folded cellular prion proteins (PrP0) to misfold; prion seeding assays mimic this process in vitro to amplify very low levels of PrPSc to levels more readily observed by conventional means. A small array of both qualitative and quantitative prion amplification assays have now been described [8-14], although with fewer researchers working to develop these assays for a finite range of target diseases and applications, their implementation has been understandably slower than was observed with PCR.

Presently, there are two fundamentally similar iterations of the prion amplification strategy – the protein misfolding cyclic amplification assay (PMCA) [7], and the real-time quaking-induced conversion assay (RT-QuIC) [13]. Both rely on a cyclical, coerced conversion of normal cellular or recombinant prion protein into the...
abnormally folded isoform. The PMCA assay provides a qualitative read-out on western blot after one or more 24–48 hour rounds of sonication, analogous to conventional and nested PCR. The RT-QuIC assay, in contrast, offers a real-time readout based on fluorescence emission over the course of 24–96 hours of shaking, much like real-time, quantitative PCR. The sources of normal prion protein, the substrate for conversion, distinguish the two assays further. Conventional PMCA relies on whole brain homogenate from a susceptible species for the PrP^C substrate (e.g. transgenic mice), while RT-QuIC makes use of recombinant PrP (rPrP), produced by bacteria or other host organisms and therefore lacking post-translational modifications such as carbohydrate moieties. This distinction between the two approaches may be responsible for the apparent absence of infectivity in amplified recombinant protein [12,15], which have otherwise been demonstrated with PMCA [16]. While the production of bona fide infectivity is certainly an asset for PMCA when studying the prion replication process, the absence of infectious PrP^Sc arising from the RT-QuIC assay could be considered an asset in a diagnostic setting by making the procedure safer for operators. The ability to standardize rPrP protein substrate concentrations in the RT-QuIC assay additionally helps with reproducibility within and between diagnostic laboratories, and would likewise be considered an important distinguishing feature. During their development, there has been some degree of cross-over between the two techniques [7,12,17,18], and moving forward it may prove beneficial to combine the two assays into a common format which takes advantage of both their strengths. At present, either assay may be run in a traditional 96-well format, allowing relatively quick analysis of samples in high volumes. Ultimately, both assays allow the experimenter to achieve log-fold amplification, each with their own important and incontrovertible distinctions in substrates fueling the reaction, amplification mechanisms, and experimental output which may make either better suited for one application or another.

A wide range of pilot studies have been conducted with both of these techniques to identify misfolded prions in various body fluids and tissues of human [19,20], animal [21-24], and even plant subjects [25]. Amplification assays have putatively identified prions in CSF [24,26], blood [27], saliva [28], urine [29, 30], feces [31-33], nasal brushings [34,35], both central and peripheral lymphoid and nervous tissues [36, 37], as well as organic and inorganic environmental samples [38,39]. Various aspects of both human and animal prion diseases have broadly been investigated using these techniques, including human Creutzfeldt-Jakob disease (CJD) [26,40], bovine spongiform encephalopathy (BSE) [41], sheep scrapie [42,43], and chronic wasting disease (CWD) of cervids [44,45]. Findings from these studies have been widely used to develop models of pathogenesis and transmission of infectious prions in their respective species.

The most extensive clinical applications of amplification assays have involved RT-QuIC testing of human cerebrospinal fluid and cervid recto-anal mucosal associated lymphoid tissue (RAMALT) biopsies for the diagnosis of CJD and CWD, respectively. In these applications, each involving multiple laboratories, there has been broad consistency in the performance of first- and second-generation RT-QuIC tests [19,26,34,46-55]. In humans, blinded RT-QuIC analyses of thousands of samples in numerous independent studies indicate diagnostic sensitivities for clinical CJD of 73–96% and specificities approaching 100%, representing a substantial improvement over other antemortem diagnostic methodologies [19,26,47-52,55,56]. A recent study has additionally indicated that clinically affected patients with sporadic CJD (sCJD) can be diagnosed with nearly 100% sensitivity and 100% specificity when considering RT-QuIC analysis of CSF, nasal brushes, or both [47]. The sensitivity of RT-QuIC may, however, be reduced when evaluating CSF samples from patients with variant CJD, Gerstmann-Straussler-Scheinker syndrome, and fatal familial insomnia [50]. These cases may simply require the incorporation of novel recombinant protein substrates, or it may indicate that different assays may be better suited for one prion disease or another, with PMCA showing some level of proficiency in amplifying each of these prion strains in a limited number of studies [40,57,58].

High levels of sensitivity and specificity have likewise been reported in cases of CWD in cervids, where the infection status of hundreds of clinical and pre-clinically affected deer and elk have been determined using RAMALT biopsies [34,53,54]. These studies have also presented a significant improvement in sensitivity over conventional antemortem diagnostic approaches. While the amplification-based diagnosis of human prion diseases, especially the diagnosis of sCJD using RT-QuIC, is becoming well established internationally in clinical prion diagnostic centers, the acceptance and timely implementation of amplification assays by agricultural agencies for the diagnosis of BSE, scrapie, and CWD has been noticeably lagging.

Each of the author’s laboratories has played a critical role in advancing amplification assays from the bench top to field applications, and each has subjectively observed limitations in both diagnostic sensitivity – difficulties demonstrating amplification in samples known to be infectious or from an infected source, and diagnostic
specificity – unexplained amplification in samples collected from disease-free sources [24,59,60]. Each group has also encountered some degree of difficulty in repeating other published experimental protocols. These limitations have made it understandably difficult to expand the application of prion amplification assays from a small number of well-characterized samples to a cohort of samples from potentially genetically diverse hosts with an unknown disease status. As a result, each laboratory has had to incorporate a range of provisions to ensure high diagnostic specificity while maintaining acceptable levels of diagnostic sensitivity.

Those provisions, detailed below, are broadly applicable to the development of all diagnostic assays, and many parallel or compliment the 2015 statement on the Standards for Reporting Diagnostic Accuracy (STARD) [61] and the UK Standards for Microbiology Investigations (https://www.gov.uk/government/collections/standards-for-microbiology-investigations-smi). The difficulties each of our laboratories have encountered and overcome while translating prion amplification assays from the lab to the field warrant further discussion to ensure that all studies using prion amplification techniques, including pilot studies, are conducted within an appropriate scientific framework to allow accurate reporting and interpretation of results and assure downstream reproducibility. Not only does future research on these diseases, and an accurate understanding of their pathogenesis and transmission, depend on reproducible experimentation, but many of our findings could and have been used to shape public policy. Important components of this framework to consider, further discussed below, include an understanding of analytical and diagnostic sensitivity and specificity, an 

**I. Estimating analytical sensitivity and specificity**

Prior to developing a robust experimental design for a project, it is important to first objectively determine the analytical specificity and sensitivity of the protein amplification assay in the laboratory. Analytical sensitivity is the upper and lower limit of detection in diluted or undiluted sample matrices, while analytical specificity describes the likelihood that amplification products are limited to the specific prion agent being evaluated. Our experience has been that substrate preparation may not be uniform between laboratories – expression levels of PrP\(^C\) found in brain homogenate preparations differ, while technical skill in preparing and purifying recombinant PrP varies. Sample matrices themselves, whether sourced from tissues, fluids or the environment, also vary in their amplification characteristics. Lastly, the abnormal prion protein itself may have variable amplification characteristics in different cellular and recombinant substrates. For these reasons it is important to include these essential preliminary experiments when initially developing the assays in a new laboratory, or when evaluating new target materials or prion targets, especially in the case of pilot studies.

Estimates of analytical sensitivity and specificity may be derived from repeated experiments using known positive and negative samples – technical replicates, which commonly include brain homogenates from a prion-infected case, or unspiked substrate for positive and negative controls, respectively. These technical controls, further discussed below, do not appropriately address the matrix-specific effects of a sample, and it would be more appropriate to consider using biological controls which are relevant to the proposed study (tissue or body fluid samples, for example).

In either case, positive and negative samples are serially diluted and subjected to amplification to determine the upper and lower limits of detection in positive sample matrices and assess the non-specific rate of conversion due to factors unrelated to the prion agent under study. Analytical sensitivity is commonly evaluated in amplification experiments (though results may go unreported), while analytical specificity is rarely examined. Ultimately, performing and reporting the results of these investigations are not only helpful for the reader and reviewer, but for the experimenter as well – to allow them to better assess the reproducibility of the assay and its specificity in that particular lab, while permitting the development of a more meaningful experimental approach.

**II. Development of an amplification strategy a priori**

**A. Amplification parameters**

Using information acquired from initial analytical experiments, researchers can more practically and effectively develop experimental amplification parameters. With an understanding of the rate at which technical
and biological negative controls demonstrate amplification, the upper limits of the number of experimental cycles can be predicted to maintain specificity. The rate at which technical and experiment-specific positive controls amplify can likewise be used to determine the minimum number of experimental cycles necessary to attain adequate sensitivity. All parameters should be firmly set ahead of time, and experimenters should avoid the practice of manipulating these parameters a posteriori under any circumstances (e.g. increasing the number of sPMCA rounds until a positive reaction is observed in samples thought to be infectious, or changing RT-QuIC threshold calculations to eliminate known false positives).

B. Selection and inclusion of appropriate controls

Two fundamentally different controls are necessary for the efficient development of prion amplification assays: technical controls (e.g. positive or negative brain homogenates, or amplification substrate alone), and experiment-specific biological controls from representative positive or negative sources (e.g. target tissues, bodily fluids, or environmental samples). Discussed separately below, both technical and biological controls may be included in the amplification strategy, depending on the questions the project is seeking to answer. These controls ultimately allow researchers to better troubleshoot assay sensitivity and specificity and interpret results. A wide selection of both positive and negative controls will additionally help researchers reduce the effects of spectrum bias – bias created by selecting samples from the extreme ends of the disease spectrum [62]. A third category of controls – unknown controls, which the experimenter is blinded to, are also discussed and may help to shed light on other sources of bias in the experimental setup.

i. Technical controls

Technical controls commonly involve samples known to have some level of PrPSc, or an absence of misfolded prion protein, based on prior examination. Positive technical controls may include brain or lymph node samples from infected patients which have previously been confirmed to be PrPSc-positive through one or more conventional methods, including immunohistochemistry (IHC), enzyme-linked immunoassay (EIA), Western blotting (WB), or bioassay. The purpose of positive (and negative) technical controls is to provide assurance that the experiment was performed correctly. Dilution series of positive samples allow for a more granular interpretation of assay reliability, and in cases where dilutions fall outside the range of expected amplification, it may allow the experimenter to reassess the experimental protocol and repeat the analysis if necessary.

Negative technical controls commonly include untreated or “unspiked” substrate material – akin to water negative controls in PCR or quantitative PCR. Untreated negative technical controls serve two major purposes – to help rule out experimental contamination and to evaluate the propensity of the substrate itself to naturally misfold in vitro. In conjunction with positive technical controls, negative controls help the researcher optimize amplification parameters, including proper temperature, pH, and ionic or adjuvant concentrations.

ii. Matrix-specific biological controls

Each sample under analysis, whether it be CSF, RAM-ALT, feces, or soil, represents a unique matrix of organic and inorganic material. The preliminary estimates of analytical sensitivity and specificity provided in experiments described above may allow the researcher to identify the optimum performance characteristics of these samples in the assay. Within each sample type, however, this matrix should also be expected to vary to some degree from source to source. Because the amplification of misfolded proteins is inherently less controlled than the templated amplification of nucleic acids, with spontaneous misfolding seen both in vivo and in vitro under various conditions, it is also helpful to have a firm understanding of the effects of sample matrix on both diagnostic specificity and sensitivity. While analytical sensitivity and specificity predict the detection limits and agent specificity, diagnostic sensitivity and specificity estimates are more concerned with the likelihood of false positive and negative reactions resulting from clinical samples with a known infection status.

Matrix effects on diagnostic specificity are best examined through biological replicates of negative samples (e.g. multiple fecal samples from several uninfected animals), which are distinct from technical replicates of a single sample. When samples are derived from animal studies, pre-exposure controls may be especially useful as biological replicates where available. Matrix-specific effects on sensitivity can likewise be examined using biological replicates of positive samples. For pilot studies, matrix spiked with brain or lymphoid tissue is a common substitute for proper biological controls, and may be the most accessible means to estimate diagnostic sensitivity and specificity – however this practice does not consider different presentations of the misfolded protein which are likely present in peripheral tissues, bodily fluids, or environmental samples. It would be more useful for both pilot and field studies to include matrix-specific biological controls with a known status (e.g. tissue homogenates previously examined in bioassay), or
otherwise rely on confirmatory assays like IHC, EIA, or WB where available, as discussed below. The number of positive and negative biological controls required is an important consideration that is partly dependent on the nature of the study, also discussed below.

iii. Unknown controls

A third category of useful controls are those with a status unknown to the experimenter. These types of controls can be effectively employed in either pilot studies or larger field studies, and may include positive and/or negative samples. A subset of unknown samples is rather easily introduced into a larger group of blinded or unblinded samples to allow the researchers to better assess sensitivity and specificity and to identify and eliminate potential points of bias.

C. Sample replicates and sample repeats

Intra-experimental replicates (e.g. a specific sample repeated in 2 or more wells on a single plate) and interexperimental repeats (e.g. a specific sample evaluated in two or more experiments) provide researchers with two important benefits — an appreciation for the repeatability of sample amplification (or absence thereof), and an opportunity to further subcategorize samples into groups: suspects, weak or strong positives, and negative or not detected, for example. Pilot studies may find repeatability information helpful, making use of both intra-experimental replicates and inter-experimental repeats, while field studies (especially prospective studies with previously demonstrated repeatability) would find subcategorization through intra-experimental replicates most useful. Many published studies have wisely made use of both intra- and inter-experimental replication, ranging from duplicate to quadruplicate. The practical repeatability of prion amplification assays is important information for researchers, however statistical data on repeatability are not always been included in publications.

D. Interpreting amplification results

Criteria for identifying positive and negative samples vary to some extent between the two major prion amplification assays. Primarily a qualitative assay, PMCA often offers a binary “seeding/no seeding” outcome, illustrated by a protease resistant band on Western blotting. Several studies have, however, constructed PMCA protocols which allow some level of quantification [45, 63]. The RT-QuIC assay, on the other hand, provides real time information which is intrinsically quantitative, or at minimum semi-quantitative, with readily available rates of amplification that clearly distinguishes this assay from PMCA [64]. RT-QuIC amplification rates are commonly calculated using an amplification threshold similar to those developed for real time PCR; a mean fluorescence level plus some consistently-applied number of standard deviations above the mean fluorescence, for example, is a starting point for threshold calculation. Studies which make use of replicates, using either PMCA or RT-QuIC, can very effectively develop firm diagnostic criteria, identifying positive or suspect samples based on the number of replicates exhibiting amplification in the case of PMCA, or the number of replicates showing amplification and their respective rates in the case of RT-QuIC.

III. Confirmatory assays

Experimental prion amplification assays have only recently begun making their way into the diagnostic arena [46,47,49,51]; as such, studies incorporating these techniques benefit from continued reliance on confirmatory assays, where available, to help support findings and allow comparisons to be drawn with conventional techniques. Commonly employed confirmatory assays include IHC, EIA and WB, which have a well-documented and widely accepted history of use in detecting prion infection [36]. In both humans and cervids, for example, a definitive diagnosis of CJD or CWD currently requires immunohistochemical detection of PrPSc in neurological or lymphoid tissues. While quite practical for tissue evaluation, these immunological assays are understood to have lower sensitivity than amplification assays, and are not useful when evaluating bodily fluids, excreta, or environmental samples – presumably a result of either a low level of abnormal prion or perhaps an undetectable form of the misfolded protein [65]. Commonly, studies which assess body fluids or excreted samples will rely on confirmation of patient disease status, however it should be remembered that patient status may not accurately reflect sample status, and a negative finding using amplification methods may simply indicate an absence of abnormal prion proteins in those particular samples. In other words, the researcher should not assume these samples to be positive simply because they were collected from infected patients. In these cases, researchers may consider bioassay for confirmation, where practical, which would additionally allow the assessment of biological relevance [66], or alternately validate with another amplification assay to allow direct comparisons between multiple approaches, for example [24].

Admittedly, one goal of the prion amplification assays currently in development is to demonstrate an enhanced sensitivity over conventional IHC, EIA, and Western
blotting [34,53], and even bioassay. Because the detection limit of prion seeding assays approaches attogram levels of PrPSc [11,35], well beyond the detection limits of traditional methods, it becomes very difficult to confirm the status of amplification-positive samples which are negative by conventional methods. Additionally, the shortage in funding available for prion research often make bioassay studies economically challenging. Researchers can, however, consider two potential avenues for demonstrating the enhanced sensitivity of amplification assays—prospective, longitudinal studies (described below), and cross-validation studies with collaborating laboratories. Without appropriate confirmation, however (bioassay or otherwise), authors are wise to refrain from providing estimates of sensitivity and specificity, as detailed in the Statistical Guidance on Reporting Results from Studies Evaluating Diagnostic Tests provided by The United States Department of Health and Human Services, instead relying on positive and negative percent agreement between experimental and conventional assays (https://www.fda.gov/downloads/MedicalDevices/DeviceRegulationandGuidance/GuidanceDocuments/ucm071287.pdf).

IV. Experimental design

Once sufficient experimentation has been undertaken to optimize assay conditions and identify any matrix-specific effects on sensitivity and specificity, after an amplification strategy has been outlined and a framework for interpreting results has been developed, and ultimately when it is determined how those results will be correlated to available information, the researcher may more effectively outline their experimental design. Factors to consider would be the nature of the study (e.g. prospective vs. retrospective), study blinding, and the number and nature of experimental controls.

A. Retrospective and prospective studies

The overwhelming majority of prion amplification studies carried out to date have been formatted as retrospective studies, making use of archived samples collected from inoculation experiments or clinical accessions. As such, the most effective retrospective studies have made use of case and control groups. Oftentimes, one of these groups is in limited number, making objective interpretation of results difficult and heightening the importance of blinded examination, further discussed below. Negative controls, for example, are commonly underrepresented in animal inoculation studies, while the rarity of prion diseases in humans often limits the number of cases available for clinical studies. When selecting samples to include in a control group, it is valuable to consider both disease-free controls as well as controls with similar clinical presentations—non-prion protein misfolding disorders (e.g. Alzheimer’s disease) or other forms of encephalitis (e.g. herpes viral encephalitis), for example. In the context of prion amplification studies, retrospective studies allow researchers the opportunity to effectively pair case samples with uninfected controls. Because they make use of archived samples, they are comparatively inexpensive and may be completed in a shorter time frame. Retrospective amplification studies are prone, however, to selection bias and in practice have suffered from an insufficient number of negative controls.

Because of the relative rarity of prion diseases, prospective studies incorporating prion amplification assays are comparatively uncommon [51,54]. Prospective studies commonly rely on natural or experimental cohorts, and are more easily undertaken in animal models of prion diseases, including chronic wasting disease in deer and scrapie in sheep. Heritable prion diseases in humans, however, may also be effectively examined using a prospective strategy when at-risk subjects with respective PRNP mutations have been identified. In contrast to retrospective studies, prospective studies are often significantly more expensive and time consuming, and require larger numbers of individuals. They may also suffer from attrition bias and an incomplete picture of clinical outcomes. Provided the researchers are blinded to the case status, prospective studies have the potential to significantly reduce testing bias, conferring greater confidence in assay results.

B. Blinded evaluation of samples

After an appropriate experimental format is identified, sample blinding is the simplest component of a robust design to incorporate into any prion amplification study. Blinded sample evaluation is an important mechanism to remove a significant level of conscious or unconscious bias from an experimental set up, and may be especially important in early pilot studies. Experimenters developing retrospective studies should consider randomly coding samples internally—using a random number generator to assign identification numbers, for example, with codes revealed upon completion of all analyses. Alternatively, prospective studies may allow for more effective blinding where the case status is not known until a final diagnosis is made available through conventional testing. In practice, unblinded PMCA or RT-QuIC studies appear to sharply outnumber those conducted blindly, though there is rarely sufficient justification for introducing unnecessary experimental bias.
C. Adequate experimental controls

The number of both positive and negative controls to include in a study is an important consideration regardless of experimental format, to allow appropriate statistical inferences to be made when evaluating study accuracy. Commonly, no more than a single positive technical control (or dilution series) and negative technical control are necessary to confirm assay function and help troubleshoot assay conditions. The number of matrix-specific biological controls required, however, will vary based on study design.

For retrospective, case-control studies, the number of positive cases will dictate the number of appropriate negative controls. In some cases, it is understandable that the number of available positive or negative controls may be limited. When evaluating suspect samples from multiple subjects in either pilot studies or advanced field studies, however, utilizing negative controls from as many sources as practical greatly helps to minimize sampling bias and more effectively supports estimates of diagnostic specificity. Likewise, it is important to have a relevant number of positive cases to more effectively estimate diagnostic sensitivity. In the rare cases where experimentation must be conducted without blinding, the number of cases and controls examined should be carefully balanced using the expected analytical and diagnostic sensitivity and specificity determined in earlier phases of the experimental development. If the number of positive cases significantly outnumber the number of available controls, or vice-versa, blinded experimentation is an absolute necessity.

For prospective studies, it is often difficult to predict the experimental outcome and determine an appropriate number of controls. Again, blinded analyses are recommended, however it is often helpful to have some number of known positive and negative control samples in the experiment. It is again important to consider the analytical and diagnostic sensitivity and specificity of the assay to more objectively estimate the number of controls to include. If calculating diagnostic sensitivity is one goal of the study, a single positive technical control may be sufficient, although a biological control would be helpful if troubleshooting is necessary. An effective starting point for negative biological controls may be 10% or more of the total number of unknown samples evaluated. As new studies are initiated, this percentage may be increased or decreased based on the diagnostic specificity reported in preliminary studies.

V. Complete reporting of results

As anyone who has handled a pipettor can attest, scientific techniques are often imperfect. Samples may be improperly labeled, aliquoted or analyzed, and experimenters might attempt to explain false positive or false negative results or simply throw out experiments altogether because of the “rare” unexpected or unexplained result. It would benefit downstream applications, however, for researchers to provide a complete summary of experimental outcomes, including the number of experiments discarded and the rationale for doing so. Were the results from a plate thrown out because of a single false positive? Did a positive control fail to amplify? Was there a known technical error? Each of these occurrences are potentially valid reasons to discard results, however if the number of experiments discarded constitute a significant portion of total experiments, it may be worth revisiting experimental development and reevaluating protocols.

Conclusions

Amplification assays for the detection and diagnosis of prion agents, although slower in their deployment than nucleic acid amplification assays, are becoming more relevant to the field of prion diseases of both animals and humans. These assays have been evolving in both form and function, although challenges remain [65]. Findings from amplification-based studies have had important implications for developing treatment options, predicting prognosis, and shaping public policy. For these reasons, it is important that these studies continue to be developed using a rigid experimental framework, including several important components of basic experimental design, in order to facilitate their interpretation.

Some of the components of this framework may simply be unattainable for some labs or experiments. Confirmatory assays are not always available, practical, or financially feasible, for example. A thorough development of experimental protocols, including adequate and appropriate controls, an a priori development of amplification strategy, blinded evaluation, and complete reporting of experimental results are, however, important considerations that any researcher and experiment can employ to reduce bias and allow for a more accurate interpretation of results. Again, the authors welcome collaborations and large sample sets may be made available which would assist in cross-validation under this framework where necessary.

Apart from the diagnosis of prion diseases, amplification assays like PMCA and RT-QuIC are expected to soon offer the ability to detect other misfolded proteins of clinical importance. In vitro amplification has been reported in cases of Parkinson’s disease [67,68] and certain tauopathies [69], with a near term goal of identifying misfolded Aβ protein [70] for the antemortem diagnosis of Alzheimer’s disease – a globally important protein.
misfolding disorder which is projected to have nearly 1 million new cases arising annually by the middle of the century according to the Alzheimer’s Association (www.alz.org). Reproducing these preliminary experiments using a larger number of unknown samples will be critical, as it has and will be for prion diseases. Because of this more “global” application of protein amplification assays, it is critical that the groundwork being laid for the PMCA- and RT-QuIC-based diagnosis of prion diseases from CJD to CWD provides an adequate foundation for these future developments.

Disclosure of potential conflicts of interest

No potential conflicts of interest were disclosed.

References

[1] Saiki RK, Scharf S, Faloona F, et al. Enzymatic amplification of beta-globin genomic sequences and restriction site analysis for diagnosis of sickle cell anemia. Science. 1985;230:1350–1354. doi:10.1126/science.2999880.

[2] Larzul D, Guigue F, Sninsky JJ, et al. Detection of hepatitis B virus sequences in serum by using in vitro enzymatic amplification. J Virol Methods. 1998;20:227–237. doi:10.1016/1066-9348(88)90126-7.

[3] Van Eys GJ, Gravekamp C, Gerritsen MJ, Quint W, et al. Detection of leptospires in urine by polymerase chain reaction. J Clin Microbiol. 1989;27:2258–2262.

[4] Rollo F, Salvi R, Torchia P. Highly sensitive and fast detection of Phoma tracheiphila by polymerase chain reaction. Appl Microbiol Biotechnol. 1990;32:572–576. doi:10.1007/BF00173730.

[5] Stefan RJ, Atlas RM. Polymerase chain reaction: applications in environmental microbiology. Annu Rev Microbiol. 1991;45:137–161. doi:10.1146/annurev.mi.45.100191.001033.

[6] Ou CY, Kwock S, Mitchell SW, et al. DNA amplification for direct detection of HIV-1 in DNA of peripheral blood mononuclear cells. Science. 1988;239:295–297. doi:10.1126/science.3336784.

[7] Saborio GP, Permanne B, Soto C. Sensitive detection of pathological prion protein by cyclic amplification of protein misfolding. Nature. 2001;41:810–813. doi:10.1038/35081095.

[8] Weber P, Giese A, Piening N, et al. Generation of genuine prion infectivity by serial PMCA. Vet Microbiol. 2007;123:346–57. doi:10.1016/j.vetmic.2007.04.004.

[9] Saa P, Castilla J, Soto C. Ultra-efficient replication of infectious prions by automated protein misfolding cyclic amplification. J Biol Chem. 2006;281:35245–35252. doi:10.1074/jbc.M603964200.

[10] Gonzalez-Montalban N, Makarava N, Ostapchenko VG, et al. Highly efficient protein misfolding cyclic amplification. PLoS Pathog. 2011;7:e1001277. doi:10.1371/journal.ppat.1001277.

[11] Chang B, Gray P, Pilitch M, et al. Surround optical fiber immunoassay (SOFIA): an ultra-sensitive assay for prion protein detection. J Virol Methods. 2009;159:15–22. doi:10.1016/j.jviromet.2009.02.019.

[12] Atarashi R, Moore RA, Sim VL, et al. Ultrasensitive detection of scrapie prion protein using seeded conversion of recombinant prion protein. Nat Methods. 2007;4:645–650. doi:10.1038/nmeth1066.

[13] Wilham JM, Orru CD, Bessen RA, et al. Rapid end-point quantitation of prion seeding activity with sensitivity comparable to bioassays. PLoS Pathog. 2010;6:e1001217. doi:10.1371/journal.ppat.1001217.

[14] Denkers ND, Henderson DM, Mathiason CK, et al. Enhanced prion detection in biological samples by magnetic particle extraction and real-time quaking-induced conversion. J Gen Virol. 2016;97:2023–2029. doi:10.1099/ijv.0.000515.

[15] Legname G, Baskakov IV, Nguyen HO, et al. Synthetic mammalian prions. Science. 2004;305:673–676. doi:10.1126/science.1100195.

[16] Ryoo C, Mays CE. Prion propagation in vitro: are we there yet? Int J Med Sci. 2008;5:347–353. doi:10.7150/ijms.5.347.

[17] Deleault NR, Lucassen RW, Suppatapone S. RNA molecules stimulate prion protein conversion. Nature. 2003;425:717–720. doi:10.1038/nature01979.

[18] Atarashi R, Wilham JM, Christensen L, et al. Simplified ultrasensitive prion detection by recombinant PrP conversion with shaking. Nat Methods. 2008;5:211–212. doi:10.1038/nmeth0308-211.

[19] McGuire LI, Peden AH, Orru CD, et al. Real time quaking-induced conversion analysis of cerebrospinal fluid in sporadic Creutzfeldt-Jakob disease. Ann Neurol. 2012;72:278–285. doi:10.1002/ana.23589.

[20] Concha-Marambio L, Pritzkow S, Moda F, et al. Detection of prions in blood. Sci Transl Med. 2016;8:370ra183. doi:10.1126/scitranslmed.aaf6188.

[21] Castilla J, Saa P, Soto C. Detection of prions in blood. Nat Med. 2005;11:982–985. doi:10.1038/nm1286.

[22] Murayama Y, Yoshioka M, Yokoyama T, et al. Efficient in vitro amplification of a mouse-adapted scrapie prion protein. Neurosci Lett. 2007;413:270–273. doi:10.1016/j.neulet.2006.11.056.

[23] Kurt TD, Perrott MR, Wilusz CJ, et al. Efficient in vitro amplification of chronic wasting disease PrPRES. J Virol. 2007;81:9605–9608. doi:10.1128/JVI.00635-07.

[24] Saa P, Castilla J, Soto C. Presymptomatic detection of prions in blood. Nat Med. 2007;13:81488. doi:10.1371/journal.pone.0081488.

[25] Farber D, Hildebrandt J, Soto C, et al. Grass plants bind, retain, uptake, and transport infectious prions. Cell Rep. 2015;11:1168–1175. doi:10.1016/j.celrep.2015.04.036.

[26] Atarashi R, Sato K, Sano K, et al. Ultrasensitive human prion detection in cerebrospinal fluid by real-time quaking-induced conversion. J Biol Chem. 2015;290:17175–17180. doi:10.1074/jbc.M115.650324.

[27] Castilla J, Saa P, Soto C. Presymptomatic detection of prions in blood. Science. 2006;313:92–94. doi:10.1126/science.1129051.

[28] Henderson DM, Manca M, Haley NJ, et al. Rapid Antemortem Detection of CWD Prions in Deer Saliva. PLoS One. 2013;8:e74377. doi:10.1371/journal.pone.0074377.
[29] Henderson DM, Denkers ND, Hoover CE, et al. Longitudinal Detection of Prion Shedding in Saliva and Urine by Chronic Wasting Disease-Infected Deer by Real-Time Quaking-Induced Conversion. J Virol. 2015;89:9338–9347. doi:10.1128/JVI.01118-15.

[30] John TR, Schatzl HM, Gilch S. Early detection of chronic wasting disease prions in urine of pre-symptomatic deer by real-time quaking-induced conversion assay. Prion. 2013;7:253–258. doi:10.4161/prion.24430.

[31] Henderson DM, Tennant JM, Haley NJ, et al. Detection of chronic wasting disease prion seeding activity in deer and elk feces by real-time quaking-induced conversion. J Gen Virol. 2017;98:1953–1962. doi:10.1099/jgv.0.008844.

[32] Pullford B, Spraker TR, Wyckoff AC, et al. Detection of PrP(Sc) in feces from naturally exposed Rocky Mountain elk (Cervus elaphus nelsoni) using protein misfolding cyclic amplification. J Wildl Dis. 2012;48:425–434. doi:10.7589/0090-3558-48.2.425.

[33] Cheng YC, Hannaoui S, John TR, et al. Early and Non-Invasive Detection of Chronic Wasting Disease Prions in Elk Feces by Real-Time Quaking Induced Conversion. PLoS One. 2016;11:e0166187. doi:10.1371/journal.pone.0166187.

[34] Haley NJ, Siepker C, Hoon-Hanks LL, et al. Seeded Amplification of Chronic Wasting Disease Prions in Nasal Brushings and Recto-anal Mucosa-Associated Lymphoid Tissues from Elk by Real-Time Quaking-Induced Conversion. J Clin Microbiol. 2016;54:1117–1126. doi:10.1128/JCM.02700-15.

[35] Orru CD, Bongianni M, Tonoli G, et al. A test for Creutzfeldt-Jakob disease using nasal brushings. N Engl J Med. 2014;371:519–529. doi:10.1056/NEJMoa1315200.

[36] Haley NJ, Carver S, Hoon-Hanks LL, et al. Seeded Amplification of Chronic Wasting Disease Prions in the lymph nodes of free-ranging cervids by real-time quaking-induced conversion. J Clin Microbiol. 2014;52:3237–3243. doi:10.1128/JCM.01258-14.

[37] Hoover CE, Davenport KA, Henderson DM, et al. Pathways of Prion Spread during Early Chronic Wasting Disease in Deer. J Virol. 2017;91:e0077–17. doi:10.1128/JVI.00077-17. Print 2017 May 15.

[38] Nichols TA, Pullford B, Wyckoff AC, et al. Detection of protease-resistant cervid prion protein in water from a CWD-endemic area. Prion. 2009;3:171–183. doi:10.4161/ pri.3.3.9819.

[39] Saunders SE, Shikiya RA, Langenfeld K, et al. Replication efficiency of soil-bound prions varies with soil type. J Virol. 2011;85:5476–5482. doi:10.1128/JVI.00282-11.

[40] Jones M, Peden AH, Prowse CV, et al. In vitro amplification and detection of variant Creutzfeldt-Jakob disease PrPSc. J Pathol. 2007;213:21–26. doi:10.1002/path.2204.

[41] Franz M, Eiden M, Balkema-Buschmann A, et al. Detection of PrP(Sc) in peripheral tissues of clinically affected cattle after oral challenge with bovine spongiform encephalopathy. J Gen Virol. 2012;93:2740–2748. doi:10.1099/vir.0.04578-0.

[42] Bucalossi C, Cosseddu G, D’Agostino C, et al. Assessment of the genetic susceptibility of sheep to scrapie by protein misfolding cyclic amplification and comparison with experimental scrapie transmission studies. J Virol. 2011;85:8386–8392. doi:10.1128/JVI.00241-11.

[43] Orru CD, Groveman BR, Raymond LD, et al. Bank Vole Prion Protein As an Apparently Universal Substrate for RT-QuIC-Based Detection and Discrimination of Prion Strains. PLoS Pathog. 2015;11:e1004983. doi:10.1371/journal.ppat.1004983.

[44] Davenport KA, Henderson DM, Bian J, et al. Insights into Chronic Wasting Disease and Bovine Spongiform Encephalopathy Species Barriers by Use of Real-Time Conversion. J Virol. 2015;89:9524–9531. doi:10.1128/JVI.01439-15.

[45] Garruto RM, Reiber C, Alfonso MP, et al. Risk behaviors in a rural community with a known point-source exposure to chronic wasting disease. Environ Health. 2008;7:31. doi:10.1186/1476-069X-7-31.

[46] Cramm M, Schmitz M, Karch A, et al. Stability and Reproducibility Underscscope Utility of RT-QuIC for Diagnosis of Creutzfeldt-Jakob Disease. Mol Neurobiol. 2016;53:1896–1904. doi:10.1007/s12053-015-9133-2.

[47] Bongianni M, Orru C, Groveman BR, et al. Diagnosis of Human Prion Disease Using Real-Time Quaking-Induced Conversion Testing of Olfactory Mucosa and Cerebrospinal Fluid Samples. JAMA Neurol. 2017;74:155–162. doi:10.1001/jamaneurol.2016.4614.

[48] Groveman BR, Orru CD, Hughson AG, et al. Extended and direct evaluation of RT-QuIC assays for Creutzfeldt-Jakob disease diagnosis. Ann Clin Transl Neurol. 2017;4:139–144. doi:10.1002/acn.3378.

[49] Orru CD, Groveman BR, Hughson AG, et al. Rapid and sensitive RT-QuIC detection of human Creutzfeldt-Jakob disease using cerebrospinal fluid. MBio. 2015;6:pii: e02451–14. doi:10.1128/mBio.02451-14.

[50] Franceschini A, Baiardi S, Hughson AG, et al. High diagnostic value of second generation CSF RT-QuIC across the wide spectrum of CJD prions. Sci Rep. 2017;7:10655. doi:10.1038/s41598-017-10922-w.

[51] Foutz A, Appleby BS, Hamlin C, et al. Diagnostic and prognostic value of human prion disease in cerebrospinal fluid. Ann Neurol. 2017;81:79–92. doi:10.1002/ana.24833.

[52] Lattanzio F, Abu-Rumeileh S, Franceschini A, et al. Prion-specific and surrogate CSF biomarkers in Creutzfeldt-Jakob disease: diagnostic accuracy in relation to molecular subtypes and analysis of neuropathological correlates of p-tau and Abeta42 levels. Acta Neuropathol. 2017;133:559–578. doi:10.1007/s12035-017-1683-0.

[53] Haley NJ, Siepker C, Walter WD, et al. Antemortem Detection of Chronic Wasting Disease Prions in Nasal Brush Collections and Rectal Biopsy Specimens from White-Tailed Deer by Real-Time Quaking-Induced Conversion. J Clin Microbiol. 2016;54:1108–1116. doi:10.1128/JCM.02699-15.

[54] Haley NJ, Henderson D, Wycoff S, Tennant J, Hoover E, Love D, Kline E, Lehmkuhl AD, Thomsen BV. Chronic wasting disease management in ranched elk using rectal biopsy testing. Prion. 2015;9:405. doi:10.1007/s12035-014-8709-6.

[55] McGuire LI, Poleggi A, Poggiolini I, et al. Cerebrospinal fluid real-time quaking-induced conversion is a robust and reliable test for sporadic creutzfeldt-jakob disease: An international study. Ann Neurol. 2016;80:160–165. doi:10.1002/ana.24679.

[56] Redaelli V, Bistaffa E, Zanussio G, et al. Detection of prion seeding activity in the olfactory mucosa of patients with...
Fatal Familial Insomnia. Sci Rep. 2017;7:46269. doi:10.1038/srep46269.

Cervenakova L, Saa P, Yakovleva O, et al. Are prions transported by plasma exosomes? Transfus Apher Sci. 2016;55:70–83. doi:10.1016/j.transci.2016.07.013.

Safar JG, Scott M, Monaghan J, et al. Measuring prions causing bovine spongiform encephalopathy or chronic wasting disease by immunoassays and transgenic mice. Nat Biotechnol. 2002;20:1147–1150. doi:10.1038/nbt748.

Hayashi Y, Iwasaki Y, Yoshikura N, et al. An autopsy-verified case of steroid-responsive encephalopathy with convulsion and a false-positive result from the real-time quaking-induced conversion assay. Prion. 2017;11:284–292. doi:10.1080/19336896.2017.1345416.

Bossuyt PM, Cohen JF, Gatsonis CA, et al. STARD 2015: updated reporting guidelines for all diagnostic accuracy studies. Ann Transl Med. 2016;4:85.

Willis BH. Spectrum bias—why clinicians need to be cautious when applying diagnostic test studies. Fam Pract. 2008;25:390–396. doi:10.1093/fampra/cmn051.

Chen B, Morales R, Barria MA, et al. Estimating prion concentration in fluids and tissues by quantitative PMCA. Nat Methods. 2010;7:519–520. doi:10.1038/nmeth.1465.

Henderson DM, Davenport KA, Haley NJ, et al. Quantitative assessment of prion infectivity in tissues and body fluids by real-time quaking-induced conversion. J Gen Virol. 2015;96:210–219. doi:10.1099/vir.0.069906-0.

Haley NJ, Richt JA. Evolution of Diagnostic Tests for Chronic Wasting Disease, a Naturally Occurring Prion Disease of Cervids. Pathogens. 2017;6:pii: E35. doi:10.3390/pathogens6030035.

Haley N, Mathiason C, Zabel MD, et al. Detection of sub-clinical CWD infection in conventional test-negative deer long after oral exposure to urine and feces from CWD+ deer. PLoS ONE. 2009;4:e7990. doi:10.1371/journal.pone.0007990.

Fairfoul G, McGuire LI, Pal S, et al. Alpha-synuclein RT-QuIC in the CSF of patients with alpha-synucleinopathies. Ann Clin Transl Neurol. 2016;3:812–818. doi:10.1002/acn3.338.

Shahnawaz M, Tokuda T, Waragai M, et al. Development of a Biochemical Diagnosis of Parkinson Disease by Detection of alpha-Synuclein Misfolded Aggregates in Cerebrospinal Fluid. JAMA Neurol. 2017;74:163–172. doi:10.1001/jamaneurol.2016.4547.

Saijo E, Ghetti B, Zanusso G, et al. Ultrasensitive and selective detection of 3-repeat tau seeding activity in Pick disease brain and cerebrospinal fluid. Acta Neuropathol. 2017;133:751–765. doi:10.1007/s00401-017-1692-z.

Salvadores N, Shahnawaz M, Scarpini E, et al. Detection of misfolded Abeta oligomers for sensitive biochemical diagnosis of Alzheimer’s disease. Cell Rep. 2014;7:261–268. doi:10.1016/j.celrep.2014.02.031.