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Review

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Application of protein misfolding amplification techniques in prion diseases

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Abstract

Prion diseases (PrDs) are fatal neurodegenerative disorders caused by the misfolding and aggregation of pathogenic prion protein (PrP^{Sc}). Traditional diagnostic methods have limited sensitivity and specificity, often requiring confirmation in the late stages or postmortem. In recent years, protein misfolding amplification techniques, including protein misfolding cyclic amplification (PMCA) and real-time quaking-induced conversion (RT-QuIC), have achieved significant breakthroughs, enabling ultrasensitive detection of PrP^{Sc} with detection limits as low as the attogram level. These methods greatly enhance diagnostic accuracy and offer the potential for early and non-invasive detection of PrDs. Their success has extended to other seeding-based neurodegenerative diseases, such as Parkinson's disease (PD), providing a powerful tool for early diagnosis, molecular pathology research, and clinical translation. Beyond diagnosis, these techniques play a crucial role in investigating strain characteristics and pathology, as well as in screening potential drugs for PrDs. They are also applied in research of public health security, including variant Creutzfeldt-Jakob disease (vCJD) surveillance, cross-species risk assessment, and environmental contamination monitoring and optimization of decontamination strategies. With exceptional sensitivity and specificity, these techniques are revolutionizing the landscape of neurodegenerative disease detection and intervention.

Keywords: Protein misfolding cyclic amplification, real-time quaking-induced conversion, prion diseases, neurodegenerative diseases

INTRODUCTION

Prion diseases (PrDs), also known as transmissible spongiform encephalopathies (TSEs), are infectious neurodegenerative disorders that affect the central nervous system (CNS) of humans and various animal species. In humans, PrDs can be classified into sporadic, genetic, and acquired forms based on their etiology. In animals, bovine spongiform encephalopathy (BSE) can manifest as sporadic (L-type and most H-type BSE)[1], genetic (a few H-type BSE cases)[2,3] and acquired (C-type BSE). The uniqueness of these diseases lies in their pathogenic mechanism, which primarily involves a conformational transformation of the host's prion protein (PrP). The normal cellular prion protein (PrP^{C}) is rich in α-helical structures, non-toxic, and readily degradable by proteases. In contrast, the pathological prion protein (PrP^{Sc}) is β-sheet-rich, forming insoluble, protease-resistant aggregates[4]. PrP^{Sc} is considered the key agent in disease transmission and pathogenesis, as it promotes the conversion of PrP^{C} into PrP^{Sc}, triggering a self-propagating cascade within the CNS.

Traditional diagnostic approaches for PrDs, particularly for sporadic and acquired forms, primarily rely on indirect biomarkers, such as hyperintensities in the cortex or basal ganglia on diffusion-weighted imaging (DWI) or fluid-attenuated inversion recovery, periodic sharp wave complexes on electroencephalography, and cerebrospinal fluid (CSF) 14-3-3 protein. However, these methods exhibit low specificity and limited predictive value in the early stages of the disease[5,6]. Current direct detection methods for pathological PrP^{Sc}, such as Western blot (WB) analysis of protease-resistant fragments, have low sensitivity, particularly in early-stage disease or peripheral samples[7]. Therefore, the development of highly sensitive and specific techniques capable of directly detecting pathological PrP^{Sc} in the early stages of disease has become an urgent priority in PrDs diagnostics.

Protein misfolding amplification techniques, primarily protein misfolding cyclic amplification (PMCA) and real-time quaking-induced conversion (RT-QuIC), have emerged to address this need. While their technical approaches and applications differ, both are based on the principle that PrP^{Sc} 'seeds' can induce the conformational conversion of PrP^{C}, enabling exponential amplification of minute amounts of PrP^{Sc}[8]. Compared to traditional detection methods, PMCA and RT-QuIC offer several orders of magnitude higher sensitivity in PrP^{Sc} detection, capable of identifying quantities as low as 10^{-18} grams[9]. Additionally, these techniques exhibit high specificity for disease-associated prion protein conformations, enabling potential detection before the onset of clinical symptoms.

This review will summarize the principles of PMCA and RT-QuIC, and focus on their applications, advantages and limitations in diagnostics, fundamental research, and therapeutic development.

PRINCIPLES OF PROTEIN MISFOLDING AMPLIFICATION TECHNIQUES

PMCA

PMCA was first developed in 2001 by Saborio and his team, simulating the 'seed amplification' process of prion protein aggregation *in vivo*[10]. The fundamental principle of PMCA involves incubating a sample containing minute amounts of PrP^{Sc} ('seeds') with an excess of normal PrP^{C} ('substrate'), allowing PrP^{Sc} to serve as a template to induce the misfolding and aggregation of PrP^{C}. Subsequently, high-frequency ultrasound pulses are applied to the sample at brief intervals, fragmenting the newly formed PrP^{Sc} aggregates into smaller pieces, each capable of acting as a new seed. Through multiple cycles of incubation and sonication, the PrP^{Sc} concentration increases exponentially[11], enabling the amplification of initially undetectable trace amounts of PrP^{Sc} to levels that can be identified by conventional detection methods. The final products can be analyzed using WB, surround optical fiber immunoassay, or other immunoassay-based techniques.

Since its development, PMCA has undergone multiple optimizations: (1) Serial PMCA (sPMCA): The amplified product is reintroduced into fresh substrate for multiple cycles, significantly enhancing amplification efficiency and sensitivity[12]; (2) Bead-assisted PMCA (PMCAb): The addition of micronized beads increases shear forces, improving amplification efficiency[13]; (3) Alternative substrates [recombinant prion protein-PMCA (rPrP-PMCA)]: In addition to traditional brain homogenate-derived PrP^{C}, recombinant PrP has been developed as an alternative substrate to reduce background variation and enhance reproducibility[14]; (4) Cross-species detection: By optimizing reaction conditions, PMCA has been successfully adapted to detect PrP^{Sc} from various prion strains and species, including chronic wasting disease (CWD) in deer and BSE in cattle[15,16].

Optimized PMCA methods have demonstrated exceptional sensitivity, with detection limits as low as 1 attogram (10^{-18} g) of PrP^{Sc}[9], meaning that even a few dozen PrP^{Sc} molecules can be detected - far surpassing the sensitivity of traditional WB assays (picogram levels). This ultra-high sensitivity has enabled PMCA to be successfully applied to a wide range of clinical and experimental samples, including CSF, blood, urine, and lymphoid tissues[17], as well as non-invasive specimens such as urine[18] and nasal mucosa[19].

However, PMCA has several problems to be addressed. De novo generation of infectious prions has been reported in PrP^{Sc}-free PMCA reactions[20,21], potentially leading to false positives. Although Cosseddu *et al.* suggested that inadvertent cross-contamination might be the cause[22], further validation of PMCA's diagnostic value and clinical applicability is necessary. Additionally, because PMCA-generated PrP^{Sc} retains infectivity, concerns regarding biohazard risks and cross-contamination must be carefully considered.

RT-QuIC

RT-QuIC was developed from PMCA by two groups working in parallel: Atarashi *et al.* applied the assay to human diagnostics[23], whereas Wilham *et al.* focused on animal testing[24]. Although RT-QuIC relies on the same seed-amplification concept as PMCA, its workflow is markedly different. It uses rPrP - typically of hamster or human origin - as a substrate, though bank vole rPrP can convert strains that resist standard substrates. Bank vole (BV) rPrP as substrate has also led to the conversion of certain prion strains, which were previously unresponsive to conventional rPrPs[25]. Except natural substrate, hybrid substrates (e.g. hamster-sheep chimera) have also yielded high sensitivity and specificity in sporadic Creutzfeldt-Jakob disease (sCJD) diagnosis (80%-89% and 99%-100%, respectively)[26-28]. The test sample, which may contain PrP^{Sc} 'seeds', is mixed with an excess amount of rPrP in a multi-well plate and incubated at 37 - 45 °C, with intermittent shaking (e.g., 60s shake/60s rest) [Table 1]. This continuous shaking provides sufficient physical energy for PrP^{Sc} seeds, if present, to convert rPrP into amyloid fibrils. Thioflavin T (ThT), added beforehand, binds β-sheet structures; its fluorescence rises as aggregation proceeds, allowing real-time tracking of the characteristic 'lag phase → exponential growth phase → plateau phase' on a plate reader[23,29].

RT-QuIC has undergone multiple optimizations to enhance its sensitivity, detection speed, and clinical applicability. The first-generation RT-QuIC (PQ-RT-QuIC) used full-length hamster rPrP (residues 23-231) as substrate and was incubated at 42 °C. Franceschini *et al.* then introduced engineered rPrP (truncated hamster rPrP, residues amino acids 90-231), raised the temperature to 55 °C, and added sodium dodecyl sulfate (SDS) to the reaction system, creating the second-generation IQ-RT-QuIC [Table 1]. Compared to PQ-RT-QuIC, IQ-RT-QuIC significantly reduced the detection time for CJD CSF samples from 55-90 h to 4-14 h[30] and converted 81% of previously negative CSF samples to positive[31]. Subsequent variants have pushed sensitivity further: eQuIC incorporates immunoprecipitation to selectively enrich PrP^{Sc} using specific antibodies[32], and IOME-RT-QuIC uses iron oxide magnetic particles to extract PrP^{Sc}[33], both excelling in biofluids with low-concentration PrP^{Sc} [Table 1].

Table 1. Reaction conditions of RT-QuIC

		PQ-RT-QuIC[24]	IQ-RT-QuIC[31]	eQuIC[32]	IOME-RT-QuIC[33]
Features		Full-length form of rPrP as substrate	Truncated form of rPrP as substrate	SDS-PBS-bead made from 15B3-coated beads (acquired from immunoprecipitation)	Superparamagnetic iron oxide beads
Substrate source		Hamster (23-231)	Hamster (90-231)	Hamster (23-231), human (23-231), hamster-sheep chimera	Hamster (90-231)
Reaction buffer	Phosphate buffer	10mM (pH 7.4)	10mM (pH 7.4)	10mM (pH 7.4)	50 mM (pH 7.4)
	NaCl	130-500 mM	300 mM	130-500 mM	320mM
	rPrP	0.1 mg/mL	0.1 mg/mL	0.1 mg/mL	0.1 mg/mL
	ThT	10 μM	10 μM	10 μM	10 μM
	EDTA	10μM	1 mM	10 μM	1 mM
Incubation temperature		42 °C	55 °C	46 °C	42 °C
Incubation time		20-68 h	24-60 h	24 h, 36-60 h (after substrate replacement)	48 h
Shaking parameters		60s shaking (700 rpm) and 60s rest	60s shaking (700 rpm) and 60s rest	60s shaking (700 rpm) and 60s rest	60s shaking (700 rpm) and 15min rest

RT-QuIC: Real-time quaking-induced conversion; PQ-RT-QuIC: first-generation RT-QuIC; IQ-RT-QuIC: second-generation RT-QuIC; eQuIC: enhanced QuIC; IOME-RT-QuIC: immunomagnetic enrichment RT-QuIC; rPrP: recombinant prion protein; SDS: sodium dodecyl sulfate; PBS: phosphate buffered saline; ThT: thioflavin T; EDTA: ethylenediaminetetraacetic acid; rpm: revolutions per min.

However, RT-QuIC also has its limitations. Sensitivity varies among sCJD subtypes, reflecting sequence mismatches between PrP^{Sc} strains and the chosen substrate. Additionally, Hermann *et al.* reported a 1.5% false-positive rate (5/371 cases) in a prospective surveillance study, with other isolated CSF RT-QuIC false positives in AD, dementia with Lewy bodies (DLB), and autoimmune encephalopathy, *etc.*[34]. Contributing factors may include spontaneous rPrP aggregation[35], *de novo* conversion[36], and excessive concentrations of salt, ThT and cleaning surfactants in the reaction mix[37].

PMCA and RT-QuIC are now the most widely used protein misfolding amplification techniques, each with distinct advantages, yet also disadvantages [Table 2]. Compared to PMCA, RT-QuIC offers higher detection efficiency, high-throughput capability, greater specificity, and lower biosafety concerns[7,29,38], making it the leading *in vitro* diagnostic method for PrDs. Although its sensitivity is usually slightly lower than multi-round PMCA, RT-QuIC still detects femtogram or even sub-femtogram quantities of PrP^{Sc}[9]. Ongoing improvements in substrate sequences, buffer composition, and incubation parameters continue to boost performance. Currently, RT-QuIC has been validated in CSF, skin, nasal mucosa, blood, urine, and feces, and the seeded-amplification concept is being translated to other protein-misfolding disorders.

APPLICATION OF PMCA AND RT-QUIC IN DIAGNOSIS OF PRDS

Application of PMCA in diagnosis

PMCA demonstrates exceptional sensitivity, making it highly promising for early and antemortem diagnosis of PrDs, particularly in variant Creutzfeldt-Jakob disease (vCJD). Independent studies by Concha-Marambio *et al.* and Bougard *et al.* showed that optimized PMCA detects PrP^{Sc} in whole-blood samples from vCJD-infected individuals with 100% sensitivity and specificity[39,40], offering a feasible approach for screening asymptomatic carriers and blood donors. Additionally, in CSF samples from vCJD patients, PMCA likewise achieved 100% sensitivity and specificity[41], further underscoring its diagnostic value in vCJD. PMCA has also succeeded with non-invasive specimens: Moda *et al.* detected PrP^{Sc} in urine from vCJD patients with 92.9% sensitivity and 100% specificity[42], while Cazzaniga *et al.* identified PrP^{Sc} in nasal-mucosa samples with 79.3% sensitivity and 100% specificity[19], highlighting its versatility across biofluids.

Table 2. Comparison of the reaction system between RT-QuIC and PMCA prion amplification assays

	RT-QuIC	PMCA
Reaction condition	Substrate: recombinant prion protein (rPrP, BV-, hamster- or human-derived) Energy: intermittent shaking	Substrate: brain homogenate (usually hamster-derived) Energy: sonication
Variation	PQ-RT-QuIC, IQ-RT-QuIC, eQuIC, IOME-RT-QuIC, *etc.*	sPMCA, PMCAb, rPrP-PMCA, PMSA, *etc.*
Standardization	Substrates prepared in laboratory with high consistency Standardized shaking frequency and time	Difficult to ensure the continuity of substrate supply and its consistency across laboratories Low standardization of sonication
Efficiency	Perform in a 96-well plate. Results are available within 24-48 h	Perform in a centrifuge tube and take longer (several days to weeks for sPMCA, 72 h for PMCAb)
Results	Quantitative analysis: Real-time monitoring of ThT fluorescence intensity The reaction product is not conformationally identical to PrP^{Sc} and lacks infectivity	Semi-quantitative analysis: WB analysis after protein digestion Infectious products are given with identical infectivity, strain properties and species specificity
Disadvantages	Sequence mismatch False positivity (1.5%), possibly related to rPrP self-aggregation, de novo conversion and excessive concentrations of reaction buffer	Spontaneous generation of PrP^{Sc}, possibly lead to false positivity Biohazard and cross-contamination

PMCA: Protein misfolding cyclic amplification; RT-QuIC: real-time quaking-induced conversion; rPrP: recombinant prion protein; BV: bank vole; PQ-RT-QuIC: first-generation RT-QuIC; IQ-RT-QuIC: second-generation RT-QuIC; eQuIC: enhanced QuIC; IOME-RT-QuIC: immunomagnetic enrichment RT-QuIC; sPMCA: serial PMCA; PMCAb: bead-assisted PMCA; rPrP-PMCA: recombinant prion protein-PMCA; PMSA: protein misfolding shaking amplification; ThT: thioflavin T; PrP^{Sc}: pathogenic prion protein; WB: Western blot.

Conventional PMCA, however, has not consistently amplified PrP^{Sc} from CSF and other biofluid samples of sCJD patients, limiting its diagnostic application in sCJD. Recent studies have shown that adjusting reaction conditions and substrate choice can yield low-level amplification from sCJD brain tissue, CSF, and nasal mucosa[43]. An improved PMCA protocol also detected PrP^{Sc} in sCJD urine samples, but with only 36% sensitivity and considerable subtype variation (VV1 and VV2 subtypes showed the highest detection rates, while MV1 and MM2 subtypes were not detected)[44]. PMCA efficiency depends on the compatibility between PrP^{Sc} 'seeds' and the reaction substrate, particularly the PRNP codon 129 genotype[38]. Further optimization is therefore required to establish ideal conditions for each sCJD subtype (MM1/MV1, MM2, MV2, VV1, VV2). Moreover, applying PMCA to non-invasive fluids such as saliva, tears, and amniotic fluid remains under investigation. If proven feasible, this would represent a major breakthrough in the early, non-invasive diagnosis of PrDs.

Application of RT-QuIC in diagnosis

In patients clinically suspected of CJD, a positive CSF RT-QuIC result is considered strong evidence for clinical confirmation. Large-scale clinical cohort studies have demonstrated that the first-generation RT-QuIC assay for detecting PrP^{Sc} seeds in CSF has a sensitivity of 73%-89%[45]. In contrast, the second-generation RT-QuIC has shown improved sensitivity, ranging from 92% to 97%[31,46], with specificity approaching 100%, highlighting its high diagnostic accuracy for detecting PrP^{Sc} in the CSF of sCJD patients[7,30,31,47].

The molecular subtypes of sCJD are defined by the polymorphism at codon 129 of the *PRNP* gene [methionine (M) or valine (V)] and the PrP^{Sc} glycotype (type 1 or type 2), resulting in distinct subtypes such as MM1 and MV1. Among sCJD patients, MM1/MV1 and VV2 are the most common subtypes and exhibit relatively high detection sensitivity. In contrast, the detection sensitivity is lower for VV1 and MM2 subtypes, with VV1 ranging from 0% to 100% and MM2 ranging from 44% to 78%[48]. Additionally, RT-QuIC shows significant variability in diagnostic value across different types of genetic PrDs. In a retrospective study of 218 genetic PrDs patients, Shi *et al.* reported RT-QuIC shows high positivity in carriers of P102L mutation (63.6%), followed by E200K (44.0%), E196A (37.5%), and T188K (25.7%), with

the lowest positivity in D178N (15.8%)[49]. This suggests that while RT-QuIC provides excellent diagnostic performance for certain PrDs subtypes, its application in rare subtypes and specific genetic PrDs remains limited. In vCJD, human-derived substrates usually failed to amplify vCJD PrP^{Sc} effectively, while BV-derived rPrP has yielded positive results, albeit with limited efficacy (one in two patients tested positive)[25]. Further optimization and refinement of RT-QuIC are necessary to improve its applicability and accuracy across different subtypes.

CSF has traditionally been the primary sample for RT-QuIC-based PrP^{Sc} detection. However, approximately 10% of patients are unable to undergo lumbar puncture due to various contraindications. This has prompted researchers to explore alternative body fluids and tissues, leading to successful PrP^{Sc} detection in skin, olfactory mucosa, tears, and blood.

In 2017, Orrú *et al.* first applied RT-QuIC to human skin samples for the diagnosis of PrDs[50]. Subsequent animal studies confirmed that PrP^{Sc} could be detected in the skin even before the onset of clinical symptoms[51], providing a theoretical basis for skin RT-QuIC in early PrDs diagnosis. Postmortem skin RT-QuIC has shown comparable sensitivity and specificity to CSF RT-QuIC in patients with PrDs[48,52]. Furthermore, Chen *et al.* demonstrated that in living patients, the RT-QuIC positivity rate at a single skin site was comparable to that of CSF RT-QuIC, and that combining samples from multiple skin sites significantly improved detection rates[53].

Due to the early and abundant PrP^{Sc} deposition in the olfactory mucosa, nasal brush RT-QuIC has gained attention as a non-invasive and convenient sample type. Studies have reported high detection rates (95%-100%) in confirmed sCJD cases, with no false positives[54,55]. Combining RT-QuIC testing of olfactory mucosa and CSF could potentially achieve near-perfect diagnostic sensitivity for clinical CJD[55]. Moreover, in the same patient samples, olfactory mucosa-based RT-QuIC exhibited a faster reaction time and higher fluorescence intensity compared to CSF-based RT-QuIC[29,56].

In 2024, Schmitz *et al.* modified the RT-QuIC by using E200K-mutant recombinant human PrP as a substrate to detect PrP^{Sc} in tears from PrDs patients, PRNP mutation carriers, and healthy controls[57]. Their findings revealed an 84% positivity rate in patients with sCJD and genetic PrDs, with sensitivity comparable to CSF RT-QuIC. Notably, PrP^{Sc} seeding activity was also detected in asymptomatic PRNP mutation carriers, suggesting that PrP^{Sc} formation may begin in the early, preclinical stages of the disease[57].

Compared to PMCA, which can directly detect PrP^{Sc} in blood, conventional RT-QuIC exhibits lower sensitivity in complex biological matrices such as blood[33]. To overcome this limitation, enhanced RT-QuIC methods have been developed, including enhanced QuIC (eQuIC) and immunomagnetic enrichment RT-QuIC (IOME-RT-QuIC). eQuIC uses antibody-mediated enrichment of PrP^{Sc} before RT-QuIC amplification, significantly increasing sensitivity[32]. Studies have shown that this method can detect PrP^{Sc} in vCJD brain tissue diluted to 10^{-14} - approximately 1 attogram of PrP^{Sc} - representing a 10000-fold sensitivity increase over standard RT-QuIC, with no false positives reported[32,58]. IOME-RT-QuIC employs superparamagnetic iron oxide nanoparticles to capture PrP amyloid aggregates, followed by magnetic separation and concentration before RT-QuIC analysis. Denkers *et al.* demonstrated that this approach improves RT-QuIC detection limit for brain-derived PrP^{Sc} by approximately 100-fold[33]. These advancements have significantly enhanced RT-QuIC's ability to detect low-level PrP^{Sc} in complex biological samples, paving the way for large-scale, non-invasive screening applications. However, blood-based RT-QuIC for sCJD diagnosis remains experimental and requires further validation and optimization.

Although RT-QuIC demonstrated extremely high specificity, it has been reported with reduced sensitivity in certain PrDs subtypes. To address this, Kraft *et al.* combined PMCA and RT-QuIC by applying the product of PMCA directly into RT-QuIC as substrate, successfully amplifying low PrP^{Sc} concentrations in muscle tissue of CWD-infected deer, whereas RT-QuIC alone yielded a markedly low amplification rate[59]. This finding highlights the potential of integrating protein amplification techniques to significantly enhance diagnostic sensitivity, particularly in samples with low PrP concentration. Therefore, RT-QuIC results should be interpreted in conjunction with clinical presentation, neuroimaging findings, and other biomarkers, and ideally used in combination with other protein amplification techniques (e.g., PMCA), to minimize the risk of misdiagnosis due to reliance on a single test result.

POTENTIAL OF PMCA AND RT-QUIC IN OTHER NEURODEGENERATIVE DISEASES

The successful research on PrDs has provided a model for other protein misfolding disorders. Many researchers are now attempting to apply PMCA and RT-QuIC to more common neurodegenerative diseases such as Alzheimer's disease (AD), Parkinson's disease (PD), and related disorders. Although the hallmark proteins of these diseases - including β-amyloid (Aβ), α-synuclein (α-syn), tau, and TAR DNA-binding protein 43 (TDP-43) - are not classically infectious agents, they exhibit self-propagating and cell-to-cell spreading behaviors similar to PrP^{Sc}[60]. Therefore, developing *in vitro* seed amplification assays for these proteinopathies promises greater diagnostic sensitivity, *in vivo* diagnosis, and even treatment monitoring.

In AD, Aβ deposition and tau neurofibrillary tangles are the primary pathological hallmarks. In 2014, Soto *et al.* introduced an Aβ-PMCA assay that incubated synthetic Aβ with patient CSF under shaking while tracking fibril formation[61]. It detected Aβ oligomers at concentrations as low as 3 fmol and differentiated AD from controls with 90% sensitivity and 92% specificity[61]. This ultrasensitive 'seed amplification' approach reveals low-level Aβ aggregates invisible to ELISA, making it attractive for early diagnosis. Nevertheless, recently developed blood biomarkers - especially plasma phosphorylated tau (p-tau 217, p-tau 181, and p-tau 231) - reach comparable accuracy and can stage disease and predict progression[62-66], potentially limiting RT-QuIC's added value for AD.

Meanwhile, significant advancements have been made with α-synucleinopathies, including PD, multiple system atrophy (MSA), and DLB. In recent years, αSyn-RT-QuIC has been widely applied to detect α-syn aggregates in CSF[67,68], skin biopsies[69,70], olfactory mucosa[71-73], duodenal and gastric mucosa[74]. Furthermore, by combining immunoprecipitation with RT-QuIC (IP-RT-QuIC), researchers have successfully amplified α-syn aggregates in blood[75] and saliva samples[76], expanding the potential for non-invasive or minimally invasive testing. In the newly proposed biological diagnostic framework for PD (SynNeurGe), pathological α-syn aggregation is a core diagnostic marker, underscoring the crucial role of *in vitro* amplification techniques such as RT-QuIC in PD diagnosis[77].

Tauopathies - including AD, progressive supranuclear palsy (PSP), frontotemporal degeneration (FTD), and Pick's disease (PiD) - feature prion-like spread of hyperphosphorylated tau. However, reliable biomarkers that reflect tau seeding in peripheral tissues are scarce. Recently, Kraus *et al.* described an improved dual-substrate tau-RT-QuIC assay capable of detecting 3-repeat (3R) and 4-repeat (4R) tau aggregates, successfully identifying 3R tau seeds in PiD patients and 4R tau seeds in PSP patients' CSF[78]. Postmortem skin tau-RT-QuIC has also shown 75%-80% sensitivity and 95%-100% specificity in AD, PSP, corticobasal degeneration (CBD), and PiD[79]. These suggest tau-RT-QuIC as a promising tool for *in vivo* diagnosis of various tauopathies. Yet, discriminating among individual tauopathies remained difficult: Though K12 RT-QuIC [using a C-terminally extended recombinant 3R tau substrate (K12CFh) as substrate] differentiated PiD (3R tau) from AD (3R/4R tau) through the quantitative differences in ThT

responses, it failed to separate mixed 3R/4R tau subtypes (e.g., AD, and primary age-related tauopathy)[80]. Future research may focus on more disease-specific substrates to further sharpen specificity.

In amyotrophic lateral sclerosis (ALS) and FTD, the primary pathological protein involved is TDP-43. Scialò *et al.* demonstrated that recombinant human TDP-43 could serve as substrate for RT-QuIC, detecting minute amounts of TDP-43 seeds (as low as 15pg) and distinguishing ALS and FTD cohorts from controls with an overall sensitivity of 94% and specificity of 85%[81]. Notably, TDP-43 RT-QuIC also yielded positive results in the CSF of a GRN mutation carrier, hinting at presymptomatic utility[81].

Taken together, these advances demonstrate that PMCA and RT-QuIC, originally established for PrDs, can be effectively extended to a wide spectrum of neurodegenerative diseases [Table 3]. While challenges remain, particularly in achieving disease-specific discrimination and optimizing substrates, these ultrasensitive protein amplification techniques already hold considerable promise for early diagnosis, differential diagnosis, and longitudinal monitoring across multiple neurodegenerative disorders.

APPLICATION OF PMCA AND RT-QUIC IN OTHER FIELDS

Investigation of prion strain characteristics

In the study of PrP^{Sc} strains, PMCA provides an *in vitro* method for replicating and characterizing different strains, offering a crucial foundation for investigating their molecular properties. By amplifying PrP^{Sc} from different phenotypic variants, researchers can assess whether the amplified products retain the characteristics of the original strains. For instance, Castilla *et al.* demonstrated that PMCA-amplified PrP^{Sc} shared similar electrophoretic mobility, glycosylation profiles, and pathogenicity with those original strains, which are derived from the brains of animals with PrDs[82]. This finding, corroborated by multiple studies[11,18,83], supports the notion that the structural conformation of PrP^{Sc} dictates strain specificity and that these conformational properties can be faithfully propagated in a cell-free system. With this capability, PMCA enables researchers to compare the biochemical characteristics, replication kinetics, and drug responsiveness of different prion strains *in vitro*. Additionally, for prion strains that are challenging to maintain in animal models, PMCA-based amplification offers an alternative approach for strain preservation and serial propagation for further analysis. In 2020, Shahnawaz *et al.* demonstrated that PMCA could be applied to detect α-syn aggregates in CSF samples to distinguish the conformational strains associated with PD and MSA, achieving a detection sensitivity of 95.4%[84]. This study further validated the potential of PMCA in the molecular classification of neurodegenerative diseases and suggested its future application in the non-invasive or minimally invasive diagnosis of PrDs. Compared to PMCA, RT-QuIC currently lacks the ability to directly discriminate between prion strains. However, it remains a valuable tool for investigating the seeding properties of novel or atypical PrDs. For example, some studies have used RT-QuIC to examine variably protease-sensitive prionopathy, comparing its pathogenic PrP^{Sc} with that of classical CJD to elucidate molecular differences and similarities between these conditions[85,86].

Research on public health security

Monitoring of vCJD

Though primarily caused by consuming meat products contaminated with BSE, vCJD has also been reported to be transmissible between individuals through blood transfusion[87] and potentially via organ or tissue transplantation from asymptomatic carriers[88]. Consequently, there is an urgent need for highly sensitive detection methods to screen biological samples and organ donors for PrP^{Sc}, thereby minimizing the risk of vCJD transmission. Concha-Marambio *et al.* demonstrated that PMCA could detect PrP^{Sc} in blood samples from vCJD patients with 100% sensitivity and specificity, requiring only a few microliters of sample for analysis[39]. Additionally, PMCA has been successfully employed to screen asymptomatic vCJD

Table 3. Applicability of RT-QuIC and PMCA in detecting other pathogenic proteins

Protein Diseases	Aβ AD	αSyn PD, MSA, DLB	Tau AD, PSP, FTD, PiD	TDP-43 ALS, FTD
Sensitivity	90% (CSF PMCA)[61]	92%-95.3% (CSF RT-QuIC)[67,68] 87.7%-94% (skin RT-QuIC)[69,70] 64.1%-96.4% (serum RT-QuIC)[75] 48%-81% (olfactory mucosa RT-QuIC)[71-73] 40.9% (duodenal RT-QuIC)[74] 68.4% (gastric RT-QuIC)[74] 83.78% (salivary RT-QuIC)[76]	75%-80% (skin RT-QuIC)[79]	94% (CSF RT-QuIC)[81]
Limitation	Superseded by blood biomarkers	Differential diagnosis between different αSyn-related diseases and their subtypes remains limited	Difficult to distinguish specific tauopathies by RT-QuIC alone	Further validation on preclinical detection is needed

RT-QuIC: Real-time quaking-induced conversion; PMCA: protein misfolding cyclic amplification; Aβ: β-amyloid; αSyn: α-synuclein; TDP-43: TAR DNA-binding protein 43; AD: Alzheimer's disease; PD: Parkinson's disease; MSA: multiple system atrophy; DLB: dementia with Lewy bodies; PSP: progressive supranuclear palsy; FTD: frontotemporal degeneration; PiD: Pick's disease; ALS: amyotrophic lateral sclerosis; CSF: cerebrospinal fluid.

carriers[39] and detect PrP^{Sc} in appendix samples[88]. These findings suggest that PMCA could be implemented for blood and blood product screening[89] to enhance transfusion safety, as well as for large-scale screening of asymptomatic vCJD carriers[90], thereby providing critical insights for public health policy. RT-QuIC, however, only amplifies PrP^{Sc} in vCJD samples with low efficacy; thus, it has not been applied to routine sample detection and monitoring of vCJD.

Assessment of species barriers and zoonotic risks

Beyond biological sample detection, PMCA and RT-QuIC have also proven valuable for assessing species barriers and zoonotic transmission risks of PrDs. Traditionally, interspecies transmission studies have relied on cross-species animal infection models, which are time-consuming and expensive. Recently, PMCA and RT-QuIC have emerged as rapid alternative methods for evaluating the cross-species transmissibility of PrDs. These techniques enable *in vitro* amplification by combining PrP^{Sc} 'seeds' and PrP^{C} substrates from different species, thereby simulating prion compatibility across species and predicting the potential and conditions for cross-species transmission[83,91,92]. For example, Barria *et al.* utilized PMCA with human PrP substrates to investigate the cross-species transmission of CWD prions from deer. Their findings indicated that after multiple cross-species passages, prion conformation could adapt in a way that may increase infectivity in humans[91]. Furthermore, *in vitro* amplification experiments demonstrated that PMCA-generated PrP^{Sc} retained the biochemical and pathogenic characteristics of the original strain, including glycosylation patterns and infectivity, while preserving species-specific transmission properties[93].

Despite the potential of PMCA and RT-QuIC for evaluating cross-species transmission risks, the abilities of various animal prion strains to cross species barriers remain debated. This discrepancy may arise from the inherent limitations of *in vitro* amplification techniques in fully replicating the complex physiological conditions of PrDs transmission *in vivo*. Future research should focus on refining PMCA conditions, expanding its application to a broader range of prion strains, and standardizing its protocols and result interpretation, which will be essential for accurately assessing species barriers, ultimately contributing to animal disease surveillance and public health management.

Detection of contaminated surface and evaluation of decontamination

PrP^{Sc} has been shown to persist on various environmental surfaces - including surgical forceps, electroencephalography electrodes, soil, stainless steel, and plant debris - while retaining its infectivity. This persistence may further enhance its environmental stability, potentially exacerbating disease transmission.

Studies have demonstrated that PMCA and RT-QuIC can detect extremely low levels of PrP^{Sc}[11,94-96], underscoring their potential for environmental surveillance. Compared to traditional bioassays using cell cultures or animal models, *in vitro* amplification techniques offer significantly higher sensitivity, shorter assay time[24,97,98], and high-throughput capabilities, making them preferred methods for detecting prion seeding activity.

Prions exhibit remarkable resistance to conventional sterilization protocols, making the decontamination of surgical instruments contaminated with PrP^{Sc} particularly challenging. Consequently, evaluating and improving decontamination procedures is critical for preventing the transmission of PrDs. Due to its ultra-high sensitivity, PMCA has been employed to detect residual PrP^{Sc} on instrument surfaces and to evaluate the effectiveness of different sterilization methods[99]. Belondrade *et al.* developed the surface PMCA (Surf-PMCA) method, a technique capable of detecting PrP^{Sc} residues equivalent to a 10^{-8} brain homogenate dilution on a single stainless steel wire, achieving sensitivity several orders of magnitude higher than traditional bioassays[94]. Based on such studies, the UK has recommended incorporating alkaline detergents and high-temperature, high-pressure sterilization for high-risk surgical instruments to maximize PrP^{Sc} clearance. Except for surgical instruments, PMCA and RT-QuIC have also been used to evaluate prion decontamination in environmental samples, such as farm soil, feeding troughs, and hospital wastewater, as well as to assess the residual infectivity of PrP^{Sc} in the environment[99].

In summary, protein misfolding amplification technologies provide an unprecedentedly sensitive approach for quantifying otherwise undetectable PrP^{Sc} contamination. These techniques facilitate the continuous refinement of prion decontamination strategies, thereby mitigating the risk of both iatrogenic and environmental transmission.

Drug screening

Currently, there are no effective treatments for PrDs; however, protein amplification techniques provide a highly efficient platform for screening anti-prion aggregation compounds. Instead of the traditional infected cell or animal models, RT-QuIC enables the rapid assessment of compounds that inhibit PrP^{Sc} formation by monitoring the extension of the lag phase and/or the reduction in ThT fluorescence amplitude[100]. This enables direct *in vitro* observation of PrP^{Sc} aggregation and inhibition, making RT-QuIC a powerful tool for evaluating the impact of compounds on prion seed propagation.

For instance, a study conducted by the Korean CDC utilized RT-QuIC to test known anti-prion compounds, including propidine derivatives, polyanions, and tannic acid. The results mirrored the PrP^{Sc} reduction observed in cell-based models. Notably, research by Hyeon *et al.* demonstrated that these compounds may either prevent PrP^{Sc} formation or promote the degradation of preformed aggregates. This highlights RT-QuIC's potential to classify compounds as either aggregation inhibitors or disassemblers, a distinction that is challenging to achieve using traditional screening approaches[101].

Furthermore, in prion-infected mouse models, skin RT-QulC seeding activity was counteracted after treatment with anti-prion compounds that extended survival time, suggesting its potential as a biomarker for therapeutic efficacy[102]. So far, high-throughput RT-QuIC screenings have already been applied to dozens of compounds, identifying promising lead molecules that significantly inhibit PrP aggregation *in vitro*, thus informing subsequent cell-based and animal studies.

In summary, PMCA and RT-QuIC offer a rapid, efficient and non-infectious approach to anti-prion drug discovery, enabling the identification and characterization of potential therapeutic agents in a controlled

environment [Figure 1]. These advances pave the way for the development of effective treatments for PrDs, accelerating progress against these devastating disorders.

CHALLENGES AND PROSPECTS

Despite the significant advancements in PMCA and RT-QuIC, several challenges remain, along with opportunities for further innovation.

First, improving the sensitivity and reliability of detecting low PrP^{Sc} concentration samples, such as blood, saliva, and urine, is crucial for achieving large-scale, non-invasive screening. While PMCA has demonstrated 100% sensitivity for detecting vCJD blood samples under laboratory settings[39-41], sCJD accounts for approximately 85% of all PrDs, and no stable or reliable non-invasive detection method has yet been established. Therefore, future research should prioritize optimizing PMCA and RT-QuIC for detecting PrP^{Sc} in non-invasive biofluids from sCJD patients, improving both sensitivity and specificity to enhance clinical applicability.

Second, current PrP^{Sc}-PMCA and RT-QuIC assays are primarily qualitative, indicating only the presence or absence of PrP^{Sc}, while quantitative analysis remains a major challenge. Srivastava et al. recently optimized end-point dilution (ED) RT-QuIC by refining dilution factors, replicate counts, and data analysis algorithms, enabling quantification of α-syn seed concentrations. The refined assay could discriminate as little as 2-fold difference in α-syn seed concentration, and detected approximately 2- to 16-fold reductions in seeding activity[103]. Applying similar approaches to PrP^{Sc} quantification could provide a valuable tool for early, dynamic monitoring of PRNP mutation carriers and help determine optimal timing for clinical interventions. Furthermore, quantifying PrP^{Sc} seeding activity could facilitate pre- and post-treatment evaluations of therapeutic efficacy, providing an objective biomarker for assessing antibody- and small molecule-based therapies, thereby advancing precision medicine and drug discovery.

Third, ongoing improvements in amplification methods may further enhance assay performance. For example, researchers have proposed replacing the ultrasound step in PMCA with a more controlled oscillation process, termed 'Protein Misfolding Shaking Amplification (PMSA)'[104], which may reduce protein conformational damage and improve amplification stability. Additionally, microfluidic chip technology has emerged as a promising tool for accelerating amplification reactions and enabling real-time detection, thereby improving efficiency while reducing operational complexity. These technological advancements will enhance the robustness and clinical practicality of PMCA and RT-QuIC, and further broaden their applications in the diagnosis and research of neurodegenerative diseases.

CONCLUSION

The invention and application of PMCA and RT-QuIC represent a major breakthrough in PrD research. These technologies enable researchers to precisely replicate the key pathogenic processes of PrDs *in vitro*, providing experimental validation for the proteinopathy hypothesis and transforming this pathogenic mechanism into highly sensitive detection methods. Clinically, PMCA and RT-QuIC have significantly improved detection rate of PrDs, enabling earlier, non-invasive diagnosis and valuable time for clinical management. In basic research, these technologies deepen our understanding of PrD transmission mechanisms, strain characteristics, and species barriers, while also accelerating the screening of potential therapeutic agents. Moreover, they play a crucial role in public health research and disease prevention and control, such as monitoring vCJD, detecting environmental contamination, and optimizing decontamination strategies. More broadly, the success of protein misfolding amplification technologies is informing work on other 'seeding-type' neurodegenerative diseases: RT-QuIC assays targeting α-syn and

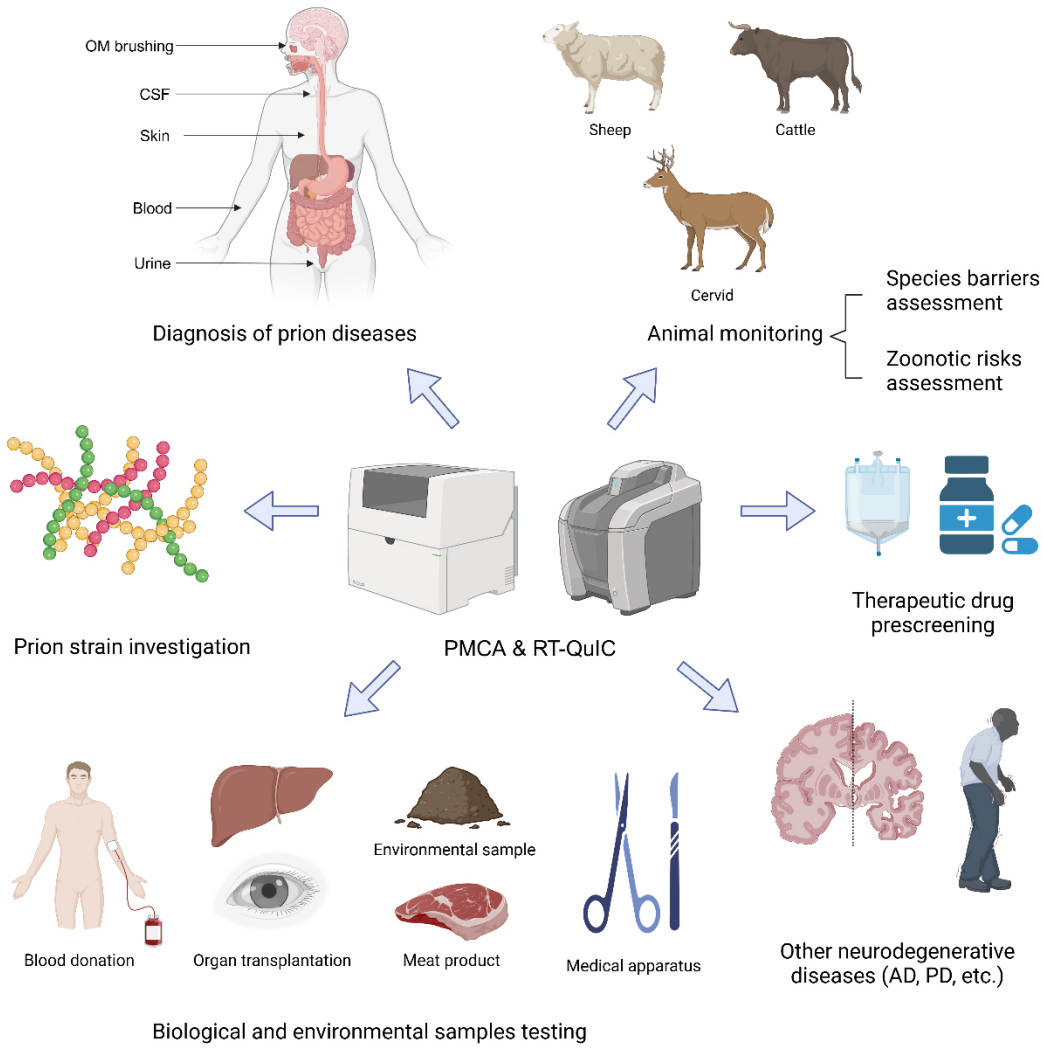

Figure 1. The applications of RT-QuIC and PMCA (Created in BioRender. LIU, R. (2025). https://BioRender.com/104rlef). They can be utilized for the diagnosis and monitoring of prion diseases, prion strain investigation, assessment of species barriers and zoonotic risks, drug screening, detection of surface contamination, and evaluation of decontamination protocols. They also hold potential as auxiliary diagnostic tools for other neurodegenerative disorders. CSF: Cerebrospinal fluid; AD: Alzheimer's disease; PD: Parkinson's disease; RT-QuIC: real-time quaking-induced conversion; PMCA: protein misfolding cyclic amplification.

tau are already reshaping the diagnostic landscape of PD and related disorders.

In summary, protein misfolding amplification technologies, with their exceptional sensitivity and specificity, form a crucial bridge between molecular pathology and clinical application, bringing new hope for tackling PrDs and other protein misfolding disorders.

DECLARATIONS

Authors' contributions

Writing and drafting: Liu R, Chen Z, Wang Y, Wu L
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Not applicable.

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Conflicts of interest
All authors declared that there are no conflicts of interest.

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