# Developing a Quick Method to Analyze Amino Acid Sequence Impact on Prion Misfolding

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**Summary**

Prion diseases or transmissible spongiform encephalopathies (TSEs) are a group of neurodegenerative diseases where the misfolding of the prion protein (PrP) is a crucial event. Based on studies in TSE affected humans and the generation of transgenic mouse models overexpressing different mutated versions of the PrP, we conclude that both wild type and mutated PrP exhibit differential propensity to misfold *in vivo*. Here, we describe a new method *in vitro* to assess and quantify the PrP misfolding phenomenon in order to better understand the molecular mechanisms involved in this process.

**Key words**

In vitro prion propagation, spontaneous recombinant prion, PMCA, rec-PMCA, protein misfolding

## 1. Introduction

Prion diseases or transmissible spongiform encephalopathies (TSEs) are a group of rare and fatal neurodegenerative disorders that affect both humans and animals. They are characterized by the accumulation of a pathogenic infectious protein called $PrP^{Sc}$ that induce abnormal folding of the normal cellular protein $PrP^{C}$.

Scrapie, affecting sheep and goats, bovine spongiform encephalopathy (BSE) in cows and chronic wasting disease (CWD) in deer are good examples of how prions are spread in nature. Humans can also be affected by various forms of TSEs: i) sporadic, i.e. Creutzfeldt-Jakob disease (sCJD) *(1)*, ii) iatrogenic, i.e. *kuru (2-3)* or vCJD *(4)*, and iii) inherited prion diseases, i.e. Familial Fatal Insomnia (FFI) *(5)*. Around the 85% of human prion diseases are grouped as sporadic, affecting worldwide approximately to

one person per year and without any currently recognized cause. There are two major possibilities considered regarding the onset of the disease: spontaneous *PRNP* gene somatic mutations or stochastic PrP protein structural changes or misfolding *(6)*.

Over 40 different mutations involved in inherited prion diseases have been described in human PrP, comprising the 15% of the human affecting prion diseases *(7)*. The most prevalent diseases enclosed in this group are: genetic CJD (gCJD), fatal familial insomnia (FFI) and Gerstmann-Sträussler-Scheinker (GSS), each one represented by distinct mutations that might trigger a spontaneous conversion from $PrP^{C}$ to $PrP^{Sc}$ *(8)*.

There are dozen of studies that, through the generation of transgenic mouse models overexpressing different mutated versions of the PrP, establish a correlation between a human *PRNP* mutation and PrP misfolding. These studies have been very valuable to understand how prions work but are strongly limited because of cost and time consuming.

In this context, we should assume that both wild type and mutated versions of PrP have a certain tendency to misfold *in vivo*. A decade ago, the $PrP^{C}$ to $PrP^{Sc}$ conversion process could be replicated *in vitro* using the Protein Misfolding Cyclic Amplification (PMCA) methodology. The PMCA requiring brain homogenates as source of $PrP^{C}$ mimicked the most important features of prions, i.e. infectivity *(9)*, species barrier *(10)* and strain phenomenon *(11)*. This technology has been of paramount importance to better understand the misfolding ($PrP^{C}$ to $PrP^{Sc}$) process. However, the need of brain homogenates has limited other potential and more accurate studies. In recent years, a new technology that requires recombinant PrP instead of brain homogenate as source of protein is allowing further studies focused on assessing and quantifying the misfolding phenomenon.

In this article we will detail the use of an adapted methodology, rec-PMCA, to evaluate the misfolding proneness of the prion protein containing different amino acid substitutions. In a similar way that the *in vitro* studies using brain-PMCA predicted the behavior of certain prions *in vivo (13)*, this new technology will contribute to understand the molecular mechanisms involved in the conversion process: transmission barrier, strain phenomenon and spontaneous generation of infectivity.

## 2. Materials

*Chemicals:*

1. Ethylenediaminetetraacetic acid (EDTA)
2. Sodium chloride (NaCl)
3. Sodium hydroxide (NaOH)
4. GnHCl (Guanidine hydrochloride)
5. N-Laurosylsarcosine sodium salt (Sarkosyl)
6. Triton X-100
7. PBS 1X (w/o Calcium, Magnesium)
8. Complete protease inhibitor cocktail (Roche)
9. Distilled water
10. Proteinase K (Roche)

*Solutions:*

1. Perfusion buffer: 5mM EDTA, PBS 1X.
2. Conversion buffer: 150 mM NaCl, 1% Triton X-100, 1 tablet of complete protease inhibitor cocktail, PBS 1X.
3. $PrP^{C}$ digestion buffer: 10% sarkosyl, 85 µg/ml PK, PBS 1X (*see* **Note 1**).
4. NuPAGE LDS sample buffer (4X) (Invitrogen).
5. Tris/Glycine/SDS running buffer (Bio-Rad).
6. Super signal West Pico (Thermo Scientific).
7. Specific primary and secondary antibodies.
8. Bleach at 20,000 ppm (commercial).
9. 1N NaOH.

*Biologicals:*

1. 4-10 weeks old PrP *knockout* (PrP-KO) mice (129/OLAxCBAxC57BL6) (*see* **Note 2**)

*Equipment:*

1. 1.5 ml centrifuge tubes.
2. 0.2 ml PCR tubes.
3. 15 ml centrifuge tubes.
4. 20G needle.
5. Slide-A-Lyzer Dialysis Cassette, 10K MWCO (Thermo Scientific Pierce).
6. PVDF trans-blot turbo transfer packs (Bio-Rad).

7. 4-15% Criterion TGX gel (Bio-Rad).
8. Surgical materials (scissors and forceps).
9. 30 ml glass tissue grinder Potter-Elvehjem (Thermo Scientific).
10. Single and multichannel pipettes (Eppendorf).
11. Plastic paraffin film.
12. Permanent markers (Sharpie).
13. Temperature probe.
14. Digital laboratory timer.
15. Criterion cell (Bio-Rad).
16. PowerPac Universal Power Supply (Bio-Rad).
17. Trans-blot turbo system (Bio-Rad).
18. Sonicator and microplate horn system S-700MPX-010 (Qsonica, LLC.) (*see* **Note 3**).
19. Sonicator tube holder and cover (Qsonica, LLC.).
20. Thermomixer comfort (Eppendorf).
21. Thermoblock dry heater (Fisher Scientific).
22. Magnetic stirrer.
23. Refrigerated centrifuge (ST 16R, Thermo Sorvall).
24. UV-Vis spectrophotometer NanoDrop 2000 (Thermo Scientific).
25. $CO_2$ mice euthanasia chamber.
26. -80º C freezer.
27. -20º C freezer.
28. Ice.
29. Dry ice.

### *3. Methods*

The methodology involved in the spontaneous generation of PK resistant (misfolded) recombinant prion protein (rec-$PrP^{res}$) by recombinant PMCA (rec-PMCA) requires two steps: i) a first step comprising the preparation of the rec-PrP-based PMCA substrate and ii) a second step concerning the unseeded *in vitro* amplification/propagation.

The whole preparation of the rec-PrP-based PMCA will be described through two different procedures (3.1 and 3.2): i) preparation of rec-PrP and ii) preparation of the helper brain homogenate. Both procedures should be performed in parallel in order to avoid withdrawal periods where the protein and/or helper molecules could be

degraded. Thus, it is worthy to stress the importance of maintaining all the compounds at 4º C before the substrate can be stored at -80º C.

Regarding the second step involving *in vitro* PMCA, similarly to what happens in a conventional PMCA, numerous variables will play an important role in the process (power and duration of sonication, temperature, etc.) increasing the importance of thorough observation of the system and the development and implementation of traceability of the process *(13-14)*.

The use of a completely prion free area and prion free instrumental is highly recommended for conducting the whole protocol. This methodology is extremely efficient amplifying pre-seeded samples making difficult to discriminate these acting as contaminants from the *bona fide de novo* unseeded samples *(15)*. We also recommend cleaning regularly the bench and the equipments using commercial bleach and/or 1N NaOH with the purpose to prevent cross-contamination of the samples *(16-17)*.

3.1. Preparation of rec-PrP as source of protein for rec-PMCA

Taking the advantage of the presence of histidines in the octarepeat region of the PrP, we recommend the use of standard protocols for the expression and histidine-affinity-based column purification of the rec-PrP (*see* **Note 4**). Importantly, the purified rec-PrP should be stored at approximately 4 mg/ml in 6M GdnHCl in aliquots (100 µl or similar) at -20ºC or lower.

1. Add 100 µl of 6M GnHCl rec-PrP (4 mg/ml) previously thawed at 4º C to 400 µl of PBS 1X and gently invert the tube several times at 4º C (*see* **Note 5**).
2. Load the 500 µl sample into a 0.1-0.5 Slide-A-Lyzer Dialysis Cassette, 10K MWCO using a 20G needle.
3. Follow 1 hour dialysis in a pre-cooled 2 L PBS container at 4º C and under continuous agitation in a magnetic stirrer.
4. Remove the sample from the dialysis cassette to a pre-cooled 1.5 ml eppendorf tube using a 20G needle. Depending on the type of rec-PrP, a white precipitation should be expected since the initial mixture increasing until the end of the dialysis.
5. Discard the naturally aggregated (likely amorphous aggregation) rec-PrP by 19,000 *g* centrifugation for 15 minutes at 4º C. Supernatant is kept at 4º C.
6. Protein concentration in the supernatant is measured by spectrophotometry (NanoDrop 2000). Around 2 mg/ml of rec-PrP is optimal for the spontaneous recombinant PMCA methodology (*see* **Note 6**).

In order to avoid spontaneous amorphous aggregation after the centrifugation (step 5), we recommend to maintain the sample at 4º C and use it as soon as possible to prepare the rec-PMCA substrate (section 3.3.). If the supernatant containing the dialyzed protein will be used after 24 h, we recommend to freeze it at -80º C and repeat the step 5 before continuing with the process described in the section 3.3.

3.2. Preparation of helper brain homogenate

Although rec-PrP might spontaneously misfold in the absence of brain homogenate complementing the substrate (see **Note 7**), the reliability of the rec-PMCA results is improved by the addition of mouse PrP-KO brain homogenate (*see* **Note 2**).

1. Euthanize a PrP-KO mouse using $CO_2$ (avoid other procedures) and perfuse the whole animal using 30 ml of PBS + 5 mM EDTA (perfusion buffer). The aim of this process is to remove completely the blood avoiding the presence of cations and certain blood components that can seriously affect the PMCA process *(18)*. A correct perfusion will keep the brain completely white before its extraction (*see* **Note 8**). We recommend performing the whole perfusion process in a prion-free animal facility in order to avoid cross-contaminations.
2. Freeze the extracted brain immediately using dry ice or liquid nitrogen and store it at -80º C.
3. Homogenate the brain at 10% (w/v) using conversion buffer and pestling at 4º C with a 30 ml glass tissue grinder Potter-Elvehjem. A standard brain will yield around 4.5-5 ml of brain homogenate.
4. Aliquot the brain homogenate in 1.5 ml tubes and centrifuge the sample at 19,000 *g* for 15 minutes at 4º C in order to discard large pieces of tissues and un-dissolved cellular membranes. Discard the pellet and use the supernatant to prepare the rec-PMCA substrate.

3.3. Preparation of rec-PMCA substrate

1. Mix by pipetting 500 µl of dialyzed rec-PrP (see section 3.1.) with 4,500 µl of PrP-KO mouse brain homogenate (see section 3.2.) in a 15 ml centrifuge tube. In order to avoid recurrent freezing-thawing, aliquot samples after proper homogenization in convenient experimental volumes, preferable in PCR tubes containing 45-50 µl of substrate. Use directly in rec-PMCA or freeze immediately substrates at -80º C before use.

2. Previous to the experimental rec-PMCA procedure, analyze the presence of rec-PrP using 1 µl of substrate (around 0.2 µg of rec-PrP) by western blotting following the protocol detailed in section 3.5. (steps 4 and 5).

3.4. Serial rounds of rec-PMCA using unseeded samples

This is the most critical procedure to generate *bona fide* misfolded rec-PrP (PK resistant rec-PrP; rec-$PrP^{res}$) spontaneously. The protocol implies to carry out serial rounds of rec-PMCA in order to refresh the rec-PrP contained in the substrate (**Figure 1)**. Depending on the propensity of the protein to misfold spontaneously, the first rec-$PrP^{res}$ positive tube will appear in any round and will continue appearing in the consecutive rounds (*see* **Note 9** and **Figure 2)**.

1. Thaw the substrate aliquots at 4º C or use them directly after their preparation as describe in section 3.3. avoiding recurrent freezing-thawing.
2. In order to obtain reliable and statistically reproducible results, use 6-8 tube duplicates per sample. We recommend to group the PCR tube strips/caps two by two in order to seal them conveniently with plastic paraffin film. This will avoid the caps open during the sonication process.
3. Place the tubes over the sonicator PCR tube adapted rack. We recommend to have the sonicator switched on and distilled water on the horn plate constantly circulating at 39ºC. The amplitude of the sonicator should be adjusted to 40-70%. This parameter will control the power of the ultrasonic wave that should work in the range of 180-220 watts. The ultrasound strength of the sonication should be adapted previously for each sonicator trying to obtain a homogeneous ultrasound (*see* **Note 10**).
4. Run a PMCA program using the following parameters: 20 s of sonication and 30 minutes of incubation per cycle at 39º C. Keep the samples at least for 24 h (48 cycles) per PMCA round (*see* **Note 10**).
5. Stop the PMCA round taking out the treated tubes and keep them at 4º C. Remove carefully the plastic paraffin film and spin down before opening the caps. We recommend cleaning the external areas of the tubes using 20,000 ppm of sodium hypochlorite and washing them before opening.
6. Perform the second round of rec-PMCA by adding (mix with intense pipetting) 5 µl from each treated tube to new thawed tubes containing 45-50µl of fresh substrate. Run the PMCA program again as described in the step 4. This serial process should be repeated according to the number of rounds that the experiment requires (*see* **Note 11**). Analyze each tube from each PMCA round

(see section 3.5.) for the presence PK resistant PrP (rec-PrP$^{res}$). Take out from the serial rec-PMCA experiment those positive tubes since they could be a source of cross-contaminations.

It is critical to keep stable the horn plate water bath temperature and sonication power during the whole serial rec-PMCA experiment in order to obtain reliable results.

3.5. PK resistant rec-PrP biochemical detection

1. Mix 10 µl of rec-PMCA-treated sample with 10 µl of digestion buffer and perform the digestion for 1 h at 42º C with shaking (450 rpm).
2. Stop the digestion by adding 1:3 (v/v) NuPAGE LDS sample buffer and heat the samples for 10 minutes at 100º C.
3. Perform the protein electrophoresis on Criterion 4-15% TGX gel (Bio-Rad) and transfer the proteins to a PVDF membrane through the Bio-Rad Trans-blot Turbo system using the manufacturer conditions.
4. Develop the membrane with the Super Signal West Pico (Thermo Scientific Pierce) after immunoblotting using specific antibodies anti-PrP.

The presence of a PK resistant 16-17 kD band will be indicative of the spontaneous generation of a misfolded recombinant protein (*see* **Note 12** and **Figure 3**). Other smaller bands, likely rec-PrP$^{res}$ fragments, can also appear although the *bona fide* (infectious) misfolded protein should weigh 16-17 kD and resist at least 50-100 µg/ml of PK in a similar way than mammalian prions.

***4. Notes***

**Note 1**: Seeded rec-PCMA has shown (unpublished data) a distinct PK sensibility for each species/strain rec-PrP$^{res}$. Thus, PK concentration (see section 3.5.) for digestion should be adapted according to the species of the rec-PrP being studied.

Although we recommend the use of 10% sarkosyl detergent for the digestion buffer, other detergents like 2% Tween, 2% NP40 work properly as well.

**Note 2:** Although we recommend the use of PrP-KO mice (129/OLAxCBAxC57BL6) *(19)*, in principle, other PrP-KO mice from any origin might be used as source of brains. Alternative to PrP-KO brains, other brain homogenate sources containing an *in vitro* inconvertible PrP$^{C}$ might be used. Thus, perfused chicken (*Gallus gallus domesticus*) brains result an adequate source of helper molecules to complement the rec-PMCA substrate as well.

**Note 3:** Previous Qsonica sonicator versions of the S-4000MPX-010 model work also adequately when the sonication power and temperature are correctly set up as described in section 3.4.

**Note 4:** rec-PrP protein purification protocols based on high-performance liquid chromatography (HPLC) couple to histidine-affinity columns also yields an adequate folded rec-PrP able to misfold spontaneously into a *bona fide* PK resistant isoform.

**Note 5**: This process is crucial and should be performed in the way that has been described exactly. Thus, a previous 1:5 dilution in PBS is necessary before starting the dialyze process. This mixture should be performed adding 1 volume of rec-PrP over 4 volumes of PBS but not in the opposite way. We believe that the first quick folding step (a quick change in the caotropic conditions from 6M GnHCl to 1.2 M GnHCl) is crutial to create misfolded molecules that will start the nucleation and seeding process along the serial rec-PMCA.

**Note 6:** The PrP concentration is a critical factor for prion propagation *in vitro (20)*. Thus, lower or higher recombinant PrP concentrations in the rec-PMCA substrate might yield distinct results in the number of PMCA rounds where the first spontaneous rec-$PrP^{res}$ appears. We recommend the use of 2 mg/ml of rec-PrP as the optimal concentration and to keep the same concentration when comparative studies are performed between different rec-PrPs.

**Note 7**: The absence of brain homogenate in the substrate significantly decreases the ability to generate recombinant $PrP^{res}$ spontaneously. This fact has been evaluated by a lower number of positive replicates and a later passage in the appearance of the first positive tube. We strongly recommend the use of PrP *knockout* mouse brain homogenate or alternative brains (see **Note 2**) to prepare the rec-PMCA substrate.

**Note 8:** If the perfusion quality has not been optimal or has been impossible to conduct, we recommend to immerse the extracted brain into a recipient containing perfusion buffer and cut it into small pieces with appropriated surgical material. This process should be performed to eliminate blood vessels and blood traces as much as possible.

**Note 9:** Since the nucleation process by which the misfolded proteins assemble to growth before acting as seed is a stochastic process, we should expect the following scenarios after performing repetitive serial rec-PMCA experiments: i) The first rec-$PrP^{res}$ positive tube does not appear always in the same round of rec-PMCA even if we repeat the experiment using the same substrate (other aliquots) and similar conditions

(the PMCA parameters cannot be maintained identical during the whole serial rec-PMCA process), ii) Not all the tubes (duplicates) in the same experiment turn positive at the same time. Although this situation could reflect that we are handling an irreproducible procedure, the proper interpretation would be that we are facing a genuine stochastic process. Thus, the use of the wild type rec-PrP as control in all the repetitive experiments is of paramount importance. In this case, we will observe differential tendencies of the different mutated rec-PrP to misfold spontaneously (**Figure 2**).

**Note 10:** Alternative PMCA parameters might work properly especially, as it was mentioned in **Note 3**, if a different sonicator is used. Thus, temperature in the water bath lower than 39º C and longer sonication periods than 20 seconds can be used. However, it is important to be aware that the results could vary significantly if the same sample is treated comparatively using different parameters. For this reason, all the changes should be assessed using wild type rec-PrP controls comparing them with the rec-PrP mutated versions in terms of their proneness to misfold. The parameters should be maintained along the whole serial rec-PMCA experiment. The trickiest parameter is the ultrasonic power, which should be calibrated manually trying to keep it with a strong and homogeneous tone during the whole experimental process.

**Note 11**: There is not information a priori about the number of rounds that a particular rec-PrP will require to misfold spontaneously. Our unpublished results indicate that most of the rec-PrP but not all are able to misfold in less than 30 rounds of rec-PMCA. There are rec-PrP species that require much less rounds of rec-PMCA than others. For that, it is crucial to use the proper control for each rec-PMCA experiment. We recommend performing a maximum of 30 rounds of rec-PMCA in those cases that a new rec-PrP is tested.

**Note 12**: In a similar way that the mammalian prion strains can appear in distinct electrophoretic migrations observed by Western blot (WB), recombinant prions might also show a differential pattern by WB. Since rec-PrP is an unglycosylated protein we should not expect so many differences comparing to what we observe in PrPs containing all the glycosylated variants. According to our unpublished results, some (may be all) rec-$PrP^{res}$ from the same species can be misfolded showing different electrophoretic patterns between 15 to 18 kD, presumably suggesting the existence of different recombinant prion strains (**Figure 3**).

**Legend of Figures**

**Figure 1: Scheme of serial rounds of rec-PMCA**. Unseeded rec-PMCA substrate based on rec-PrP and PrP-KO brain homogenate is subjected to a round of rec-PMCA (cycles of sonication-incubation steps). After 24-48 h occurring the first round, a 1:10 dilution is performed to re-fresh the rec-PrP. This process is repeated serially and the samples analyzed for the presence of PK resistant rec-$PrP^{res}$.

**Figure 2: Comparative of misfolding proneness between wild type (wt) and L108I mouse rec-PrP using serial rec-PMCA.** Two different substrates based on wt and L108I mouse rec-PrP were subjected to five independent experiments of 12 rounds serial rec-PMCA. Each experiment contained 4 tube duplicates. Legend indicates the percentage of positive tubes that turned rec-$PrP^{res}$ positive after their PK digestion.

**Figure 3: Biochemical analysis of different recombinant mouse prions.** Unseeded mouse brain homogenates from different serial rec-PMCA experiments were digested with 85 µl/ml of PK for 1 h at 42º C. Different electrophoretic patterns are observed. Human $PrP^{res}$: Human brain from a sCJD affected patient was used as control after its digestion with 85 µl/ml of PK for 1 h at 42º C. rec-PrP: PK undigested mouse rec-PrP. Control: PK undigested mouse brain homogenate.

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