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Optimization, adaptation and application of protein misfolding cyclic amplification to detection of prions in blood plasma

by

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ABSTRACT

The PMCA assay was optimized for adaptation to low level detection of PrP^{Sc} in hamster plasma. Evaluation of numerous key variables of the PMCA assay led to an optimized protocol capable of ~3 $\log_{10}$ amplification after 32 cycles (two 16 hour rounds). When commercially purchased normal hamster plasma was added to the PMCA reaction an accentuation in PrP^{Sc} amplification was observed (>6.75 $\log_{10}$ after 32 cycles). Only con-specific plasma appeared to enhance the conversion of PrP^{C} to PrP^{Sc}, suggesting that a species-specific co-factor may be involved in assembly of protein aggregates. Serial PMCA in the presence of low level (10%) contiguous conspecific plasma resulted in the generation of *de novo* PrP^{Sc} after several rounds of PMCA. Although plasma significantly accentuated PrP^{Sc} amplification by PMCA, the formation of *de novo* PrP^{Sc} interfered with the ability of using the PMCA assay to detect prion infections in hamsters experimentally infected with 263K scrapie.

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aa	amino acid
Ab	antibody
ACC	autocatalytic conversion assay
ALS	amyotrophic lateral sclerosis
ag	attogram
aPMCA	automated PMCA
BBB	blood brain barrier
BH	brain homogenate
BSE	bovine spongiform encephalopathy
CB	conversion buffer
cBSE	classical BSE
CCAC	Canadian Council of Animal Care
CDI	conformation dependent immunoassay
CEA	Commissariat à l'Énergie Atomique
CFIA	Canadian Food Inspection Agency
CJD	Creutzfeldt-Jakob disease
CNS	central nervous system
CSF	cerebrospinal fluid
CWD	chronic wasting disease
dil'n	dilution
DNA	deoxyribonucleic acid
dpi	days post inoculation
EDTA	ethylenediaminetetraacetic acid
ELISA	enzyme-linked immunosorbant assay
EPA	epitope protection assay
ERAD	endoplasmic reticulum quality control system
EtOH	ethanol
FFI	fatal familial insomnia
fg	femtogram
FSE	feline spongiform encephalopathy
g	gram
GdnHCl	guanidinium hydrochloride
GSS	Gerstmann-Sträussler-Scheinker disease
HCl	hydrochloric acid
HPrP	human PrP
hr	hour

IBH	infectious brain homogenate
IC	intracranial
IHC	immunohistochemistry
Inc.	incorporated
IP	intraperitoneal
iPCR	immuno-PCR
iq-RT-PCR	immunoquantitative-real time-PCR
KCl	potassium chloride
kD	kiloDalton
KH_2PO_4	potassium phosphate monobasic
L	litre
LD_{50}	lethal dose killing 50% of animals
LDL	low density lipoproteins
min	minute
mL	millilitre
mM	millimolar
MW	molecular weight marker
NaCl	sodium chloride
NaOH	sodium hydroxide
Na_2HPO_4	disodium phosphate dibasic
NBH	normal brain homogenate
ND	no data
OLF	Ottawa Laboratory Fallowfield
PAGE	polyacrylamide gel electrophoresis
PBS	phosphate buffered saline
PBST	phosphate buffered saline with Tween
PCR	polymerase chain reaction
pg	picogram
PK	proteinase K
pM	picomolar
PMCA	protein misfolding cyclic amplification
poly A	polyadenylic acid
PrP	prion protein
PrP^{C}	cellular prion protein
PrP^{Sc}	infectious prion protein
PrP^{sen}	infectious prion protein (PrP^{Sc}) destroyed by PK digestion
PrP^{res}	resistant prion protein (PrP^{Sc}) not destroyed by PK digestion
PVDF	polyvinylidene difluoride
QuIC	quaking induced conversion

RNA	ribonucleic acid
rHaPrP	recombinant hamster PrP
rPMCA	recombinant PMCA
rPrP	recombinant PrP
SAF	scrapie associated fibrils
saPMCA	serial automated PMCA
Sc237	scrapie hamster line
sCJD	sporadic CJD
SDS	sodium dodecyl sulfate
SOFIA	surround optical fiber immunoassay
sPMCA	serial PMCA
sec	second
Tg_{cer}	cervidized transgenic mice
TME	mink spongiform encephalopathy
TSE	transmissible spongiform encephalopathy
vs	versus
WB	Western blot
uL	microlitre
USA	United States of America
V	volts
v/v	volume/volume

1 INTRODUCTION

Transmissible spongiform encephalopathies (TSEs) pose unique challenges for diagnosis, prevention and treatment compared to other transmissible diseases. Death is an inevitable outcome of TSE infection, and a significant number of human deaths have been associated with various forms of TSEs. These diseases have also been particularly devastating to wildlife and the agricultural industry. Therefore the development of a sensitive TSE ante-mortem diagnostic test has become the focus of numerous research groups.

1.1 Background

The degenerative disease of sheep known as scrapie was described as early as the 18th century. Bjorn Sigurdsson proposed the term 'slow virus', in 1954 (62,217), to explain the lengthy incubation period associated with both scrapie and visna, diseases of the central nervous system (CNS). In 1959, William Hadlow observed the histopathological similarities between scrapie and kuru, a disease of primitive tribes in Papua New Guinea where the source of infection arose from the ritualistic cannibalism of infected brain material (97). Kuru was thus identified as a CNS disease associated with a slow virus (130). The relatedness of kuru and Creutzfeldt-Jakob disease (CJD) was subsequently confirmed by the transmissibility of both diseases to chimpanzees in 1966 and 1968, respectively, by Carleton Gajdusek (79,80), for which he was awarded a Nobel Prize in 1976. The pathological characteristic of tiny holes (*i.e.*, vacuoles) giving a spongy appearance in brain tissue, was common among individuals infected with kuru and CJD and in animals infected with scrapie. In conjunction

with the known transmissibility of these diseases, the term transmissible spongiform encephalopathy was designated for this class of infectious diseases.

Until 1982, most scientific researchers believed the etiological agent of TSEs to be a slow virus due to the progressive and predictable pattern of disease onset and development (23). However, several unique characteristics about the diseases were distinct from those caused by viruses. First, TSE diseases were characterized by a lack of an immunological response (81), with little or no inflammation associated with progressive neuronal degeneration (188,258). Second, the infectious agent did not appear to be encoded by nucleic acids, since high doses of ultraviolet light, gamma radiation or treatment with nucleases, which normally destroy infectivity of viruses, did not inactivate TSE agents (3,84,138). Third, the heterogeneous size of the TSE agents were unlike viruses whose sizes are often larger and more predictable (67). Fourth, there was a notable absence of a virus-like structure consistent with a typical microorganism present in the CNS of infected individuals (67).

Since the etiological agent associated with TSE diseases appeared to be quite distinct from viruses, two hypotheses emerged to explain the nature of the infectious agent; the ‘virino hypothesis’ and the ‘protein-only hypothesis’. The virino hypothesis maintained that the infectious agent was a small nucleic acid that was not translated or easily inactivated and whose expression was dependent on a host-coded protein to form an infectious unit (44,128). The protein-only hypothesis argued that the infectious agent was a self-replicating host protein, given the term prion. The theory proposed that prions exist in at least two

molecular forms, a properly folded protein (PrP^{C}) or a misfolded protein (PrP^{res} or PrP^{Sc}), and that the infectious properties of this host prion protein coincide with the misfolded conformational state propagating disease through the recruitment and template-directed misfolding of normal prion protein (184). Although much controversy still exists around these two theories, the protein-only hypothesis is the widely accepted theory. Stanley Prusiner was awarded the 1997 Nobel Prize in Medicine for his discovery and the theory that prions reflected a new biological principle for infection.

1.1.1 *Human and Animal TSEs*

Human prion diseases include CJD, Gerstmann-Sträussler-Scheinker disease (GSS) and fatal familial insomnia (FFI). Sporadic CJD (sCJD) is the most common human prion disease accounting for ~85% of the cases (119), while 10-15% of cases are familial or inherited (*i.e.*, GSS, FFI) and ~1% have been linked to iatrogenic or foodborne exposure (76,85,108,109,119). Although all forms of human prion diseases may be acquired through iatrogenic exposure, generally only two diseases are recognized as acquired (*i.e.*, transmissible) in humans. Transmissible human prion diseases (*i.e.*, human TSEs) are limited to kuru, identified in tribes in New Guinea prior to the 1960s, and variant CJD (vCJD) first reported in 1996 (239).

Prion diseases are not unique to humans, but also occur in animals. Scrapie occurs in sheep and goats, bovine spongiform encephalopathy (BSE) in cattle, chronic wasting disease (CWD) in cervids, transmissible encephalopathy in mink (TME) and feline spongiform encephalopathy in cats (FSE). Animal prion diseases are typically transmissible, but may have arose sporadically in cattle

(50,119). Table 1.1 lists the currently recognized human and animal prion diseases along with their classification (*i.e.*, inherited, sporadic, acquired).

In all cases of human and animal TSEs, spongiform brain degeneration represents a hallmark characteristic of the disease. Progressive neuronal degeneration leads to a number of clinical symptoms associated with disease progression. Depending on the prion disease, various clinical signs may be displayed. Typically in humans, TSEs are recognized clinically as rapid progressive dementia, ataxia (loss of muscle coordination), or progressive blindness, but can only be confirmed with certainty by autopsy.

Animals with prion diseases display various behavioural changes dependent on the species. Infected cattle show a variety of different symptoms, including nervous or aggressive behaviour, abnormal posture, lack of coordination or difficulty in rising from a lying position, decreased milk production and weight loss despite an increased appetite (36). Scrapie is characterized by the scraping of fleece and stumbling in sheep (127). In CWD, infected cervids appear to wither away and have symptoms associated with behavioural and physiological changes such as unsteadiness and excessive salivation (118). Domesticated cats and captive felids (in zoos) experience ataxia, with more timid or more aggressive behaviour, and hypersensitivity to sound and touch (180,215,260). Ranched raised mink display locomotor disturbances and excitability, followed by ataxia in the hind quarters with progression of disease (13).

Several other disease-associated characteristics are unique to TSEs, including an extremely long incubation period without the presence of clinical symptoms (238), followed by a clinical phase without an identifiable inflammatory process. The cumulative evidence supporting the hypothesis that a host-derived protein may be ultimately responsible for disease (31), makes these diseases difficult to diagnose, prevent (*i.e.,* vaccine) or treat (*i.e.,* drug-based therapies). Currently no prevention or treatment strategies are available, and therefore all prion diseases are inevitably fatal.

Table 1.1 Classification of human and animal prion diseases.

	Inherited (genetic)	**Sporadic**	**Acquired**
Human			
Gerstmann-Sträussler-Scheinker (GSS)	✓		
Fatal Familial Insomnia (FFI)	✓		
Creutzfeldt-Jakob Disease (CJD)	✓	✓	
variant Creutzfeldt-Jakob Disease (vCJD)			✓
Kuru			✓
Animal			
Scrapie			✓
Chronic Wasting Disease (CWD)			✓
Bovine Spongiform Encephalopathy (BSE)		✓	✓
Feline Spongiform Encephalopathy (FSE)			✓
Transmissible Mink Encephalopathy (TME)			✓

1.1.2 *Protein structure and function*

In mammals, normal cellular prion protein (PrP^C) is encoded by the *prnp* gene (168). PrP^C is a glycosylphosphatidylinositol (GPI)-linked membrane protein, highly expressed on neuronal cells of the CNS (225). It is composed of approximately 220 amino acids (dependent on species), with a variable octarepeat region, a single disulfide bond, and a predominantly α-helical confirmation (174).

PrP^C is essential for prion disease progression, as shown by Bueler *et al.* (33), where PrP^C knockout mice inoculated with scrapie prions failed to exhibit disease (45).

The normal cellular protein is believed to be anchored at the cell membrane, with a half-life at the cell surface of 5 hours, after which the protein is internalized through a caveolae-dependent mechanism and degraded in the endolysosome compartment (43). Conversion to PrP^{Sc} likely occurs during this internalization process (67,68) and the α-helical coil structure (PrP^C) is refolded into β-sheet rich (PrP^{Sc}) conformation (174). It has been speculated that a malfunction of the endoplasmic reticulum quality control system (ERAD) may assist misfolding (64,65,106). Clarification of the normal function of PrP^C may help explain why the typical mechanism of protein quality control fails to recognize and eliminate the misfolded form of the protein.

Both PrP^C and PrP^{Sc} have identical amino acid sequences, lack nucleic acids, and have no known primary protein structural differences (40), but are dramatically different in their biophysical properties (Table 1.2). PrP^{Sc} not only differs from PrP^C in quaternary structure, but it is more resistant to proteases, dehydration, alcohols, radiation, fixatives, heat (*i.e.*, autoclaving) and pH, and is insoluble in the presence of non-denaturing agents (151,168,191). The reduced solubility of PrP^{Sc} results in protein aggregation (174) and PrP^{Sc} aggregation is associated with prion disease (261).

The wide spread tissue distribution and expression of PrP^C on many cell types suggests that PrP^C is not solely restricted to neuronal function (234). The

specific role of this protein has not yet been identified, however copper binding has been speculated (27,155) which may play a role in the kinetics and pathobiology of TSEs (154). Evidence in favor of a direct association of PrP^C with copper stems from work by Quaglio *et al.* (192), where the addition of copper assisted in the conversion of PrP^C to a partially protease resistant form. Pauly *et al.* (179) also demonstrated that copper increased PrP^C endocytosis and thereby affected PrP trafficking. Notably the observation that PrP knockout mice have substantially less copper associated with their brains compared to wild type mice also suggests a strong linkage between copper and PrP (27,154). The similarities and a potentially overlapping function with other members of this family of proteins (*i.e.*, the Shadoo protein [a CNS glycoprotein]) may aid to further elucidate PrPs' true function (249).

Table 1.2 Comparison of the biophysical and chemical properties of PrP^C and PrP^{Sc}.

Protein Properties	PrP^C	PrP^{Sc}
Secondary structure	'properly folded'	'misfolded'
	α – helices	ß – sheets
Infectivity	noninfectious	infectious, pathogenic
Aggregation status	monomer, dimer, oligomer	oligomers,aggregates, forms fibrils
Solubility	soluble (hydrophilic)	insoluble (hydrophobic)
Protease sensitivity	susceptible	partially, but generally highly resistant
Size	33-35 kD	27-30 kD[a]

[a] after PK digestion

1.1.3 *Disease classification and host genotype*

Prion diseases are recognized as one of three types: inherited, sporadic, or acquired (Table 1.1). Susceptibility to inherited prion diseases involves a

mutation in the *prnp* gene and can thus be diagnosed using molecular markers. GSS and FFI are both hereditary human prion diseases. CJD is also a hereditary prion disease, but individuals can suffer from the disease in the absence of a mutation in the *prnp* gene. CJD in these patients is classified as either sporadic CJD (sCJD), with no known cause, or acquired (vCJD), which has been typically linked to the consumption of prion infected meat (of cattle) or iatrogenic exposure. Thus, sporadic and acquired TSEs are much more difficult to diagnose.

In humans, a common PrP polymorphism at residue 129 (methionine/methionine [M/M] or valine/valine [V/V]), is a key determinant of genetic susceptibility to acquired or sporadic prion diseases, with disease occurring mainly in homozygous individuals (50,51,172). In animal prion diseases, homozygous sheep with alanine-arginine-arginine/alanine-arginine-arginine (ARR/ARR) at codons 136, 154, 171 respectively were considered clinically resistant to conventional scrapie (112). However, the discovery of atypical scrapie, occurring in ARR homozygous sheep, was established and classified as distinct from conventional scrapie by diagnostic analysis. In the case of CWD, genetic susceptibility is influenced by a variety of polymorphisms in the *prnp* gene and vary depending on species. In elk, there is speculation that alleles encoding either methionine/leucine (M/L) or leucine/leucine (L/L) at codon 132 may lengthen the incubation period of disease (167,256). Mule deer heterozygous with serine/phenylalanine (S/F) or homozygous with phenylalanine (F/F) at codon 225 appear more resistant to disease (116), whereas in white-tailed deer polymorphisms at 3 codons (95, 96 and 226) seem to influence disease resistance

with glycine/96/serine (G96S) displaying moderate resistance (117). In cattle, susceptibility to BSE does not appear to be linked to a PrP codon polymorphism, but may be associated with modification of the PrP gene itself (*i.e.*, additional repeat regions, base pair insertions and/or deletions). For example, variations in two bovine *prnp* regions (*i.e.*, 23-bp promoter, 12-bp intron 1) have been speculated to increase susceptibility to classical BSE (cBSE) in some, but not all bovine species (32). Currently no such modifications have been identified for atypical BSE (209). The recognition and classification of atypical BSE was a consequence of worldwide increased BSE testing to minimize and eliminate foodborne exposure to these zoonotic diseases (54). Prion disease in mink has been recognized since 1947, yet the genetic susceptibility factors for both TME and FSE remain unknown due to their low occurrence since the ban of bovine (spleen and CNS tissue) and sheep material in pet food in 1990 (215).

1.1.4 *Distribution of infectious prions in tissues and fluids*

Although brain degeneration and spongiform appearance is the hallmark characteristic of all species displaying prion disease, infectious prions have been shown to be distributed to other bodily tissues and fluids. The extent of tissue distribution seems to be dependent on species and prion disease (Table 1.3). A comparison of the tissue distribution of cattle with that of sheep and cervids demonstrates the pathophysiological differences between the diseases. Infectivity in cattle (BSE) is limited primarily to the brain, spinal cord, and retina, but also associated with the distal ileum and tonsil (49,66,235,243,254). Scrapie and CWD infectivity occurs in these same tissues but also in numerous other tissues (Table 1.3).

Due to the extended tissue distribution in cervids and sheep, it is not surprising that infectious prions have subsequently been found in various bodily fluids associated with both secretory and excretory systems. Although infectivity of faeces, urine and saliva has only been confirmed recently (123,147,229), as early as 1962 Pattison *et al.* (177) believed that the lack of infectivity associated with these fluids was quite probably related to insufficient quality or unsuitable form to produce clinical disease and not necessarily that they were non-infectious. Nevertheless it took until 1998 for Brown *et al.* (26) to first confirm the infectious nature of blood by intracranial rodent inoculation, followed by the successful blood transfusion of infectivity in sheep by Hunter *et al.* (111) in 2002. Infectivity associated with saliva, urine and faeces in animals has only been confirmed within the last three years (123,147,229).

Determining the tissue and fluid distribution associated with human prion diseases is much more complicated. It is unethical to knowingly transmit prion diseases within humans, therefore all evidence about human tissue and fluid distribution has been surmised from non-host specific animal experimentation or direct detection of PrP^{Sc} in infected individuals. In addition, epidemiological linkages between clinical patients (*i.e.,* from surgical procedures and medical records) have also provided evidence of transmissibility of human-associated TSEs in bodily tissues or fluids. Based on the most widely studied human prion disease, CJD, it is clear that the lymphoreticular tissue and fluid distribution is present in vCJD, but absent in cCJD (245) (see Table 1.4). Infectious prions have only been confirmed in dura mater of the brain (259), cornea of the eye (69),

spinal cord (99), appendix (108), tonsil (107), adrenal gland and thymus (247), rectum (246), pituitary gonadotropic hormone (47), growth hormone (183) and blood (142) of humans.

Table 1.3 Animal tissues and fluids/excreta with confirmed infectivity and/or PrP^{Sc} deposition.

Tissue	**Species displaying confirmed infectivity and/or PrP^{Sc} deposition**
Brain	sheep (98), cervids (223,256), cattle (103,114,243), rodent (71,189)
Spinal cord	sheep (98), cervids (223,256), cattle (103,114,243), hamsters (148), rodent (71)
Retina	mule deer (223,256), cattle (103)
Eye lid (3rd)	sheep (165)
Lymph nodes: Retropharyngeal	sheep (98), cervids (223,256)
Peripheral	sheep (98), deer (223,256)
Spleen	sheep (98), rodent (126,189)
Tonsil	sheep (98,211,241), deer (255), cattle (254)
Salivary glands	sheep (177)
Ileum	sheep (98), cattle (253)
Peyer's patch	sheep (105)
Muscle: Skeletal Cardiac Tongue	deer (5) cervids (115) rodent (158)
Placenta (or associated tissue)	sheep (169,178,193,240)
Skin	sheep and hamster (236)
Fluid/excreta	
Cerebral spinal fluid	sheep (98)
Placental discharge	sheep (62,63,144)
Blood	sheep (111), deer (147), rodent (26)
Milk	sheep (135)
Saliva	deer (147)
Urine	rodent (123)
Faeces	deer (100,101,229)
Rectal mucosa	sheep (72,88,89), elk (221)

Table 1.4 List of infectious tissues and fluids associated with humans infected with cCJD or vCJD.

Tissue	cCJD	vCJD
Brain	✓	✓
Spinal cord	✓	
Eyes		✓
Tonsil		✓
Adrenal gland		✓
Lymph nodes		✓
Appendix		✓
Rectum		✓
Fluids		
Hormones (pituitary)		✓
Blood		✓

1.1.5 *Mode of transmission*

Like many infectious diseases, prions appear to have multiple routes of transmission. In humans, exposure routes have thus far been limited to transplantation or transfusion of CJD infected tissue or foodborne exposure through the consumption of BSE-infected beef products. In animals the predominant mode of transmission of prion diseases have been clearly identified for some species, such as, BSE, FSE and TME, all linked to the consumption of contaminated feed with infected prion tissue (153,215,257). In others animal TSEs the infective nature is contagious and spreads from animal to offspring (*i.e.*, vertical transmission) or from animal to animal (*i.e.*, horizontal transmission) by either direct or indirect modes. The most recognized example of vertical transmission is scrapie, described as primarily maternal and not highly infectious, but transmission is also partially associated with horizontal routes (63). Placental infectivity has been documented for scrapie (169,178,193), but Tuo *et al.* (240) detected PrP^{Sc} only in tissues physically separated from the fetus, suggesting

transmission may occur post-birth. The acquisition of prions post-birth is reinforced by the large accumulation of PrP^{Sc} detected in the Peyer's patch of lambs (105) and other studies that suggest that infection occurs via the alimentary tract (4,98). Infectivity can be transmitted to the environment via placental discharge, and therefore it is possible that other sheep in the flock may also become infected (62,63,144).

In the case of CWD, placental infectivity or maternal transmission have yet to be confirmed, but it is believed that according to epidemiologic and mathematical models these modes of transmission alone would not sustain the current epidemics. The data suggests other routes of horizontal transmission must occur (152). Miller *et al.* (153) investigated several routes of horizontal transmission in mule deer; direct contact between live deer, exposure to areas where an infected carcass has decomposed, and indirect exposure to residual excreta where infected animals had previously been housed. Each exposure scenario resulted in at least one deer becoming infected within a one year period. Horizontal transmission of CWD is further supported by confirmed infectivity of saliva, urine and faeces via transgenic mouse bioassay (100,101,147,229). Tamguney *et al.* (229) reported that the infectious dose in faecal excreta was considerably lower than in brain tissue (measured from the same deer at disease onset), however low level shedding over a lifetime may result in similar levels of infectivity (as measured in the brain) being passed to the environment.

1.1.6 *Resistance and persistence*

Prions are extremely resistant to degradation or disinfection and are able to withstand preservation in fixatives, heat, combustion, ultraviolet light and gamma radiation (84,138). For this reason, prion research is restricted to laboratories where dedicated prion handling facilities exist or where these agents can be contained. The generated waste material, along with equipment and tools used in these laboratories are then exposed to extreme conditions, such as strong sodium hypochlorite solutions, hot solutions of sodium hydroxide, or autoclaving at 134°C for 60 minutes, that are necessary to inactivate prions (231). Health care providers and practitioners must also be aware of the resistance and persistence of prions. Neurosurgical equipment used on an pre-clinical CJD patient led to the subsequent transmission to two other patients (25), confirmed by the implantation of the instrument tip into the brain of a chimpanzee, proving that the infectious agent had remained intact for several years through numerous disinfection and sterilizations (19,83). The knowledge that prions bind with high affinity to steel and plastics (252,265) means that the universal precautions employed by hospitals are often not extensive enough to eliminate the risk of iatrogenic exposure. The increased rate of sCJD has been speculated as related to the increased number of surgical procedures (52,248), yet harsh decontaminations procedures outlined for prion removal are often too damaging and impractical for most surgical instruments (228).

The environmental resiliency of prions to decomposition is also remarkable. Brown and Gajdusek (30) observed detectable infectivity 3 years

after the supernatant from a scrapie infected hamster brain was buried in soil. The persistence of prions was also demonstrated in Iceland when scrapie-free flocks were reintroduced to scrapie sheep pastures left uninhabited for 3 years, with disease subsequently developing in the reintroduced flock (173). Decay of infected carcasses or burial of diseased and infected material have also been shown to transmit disease (153) and PrP^{Sc} has been shown to bind with high affinity to soil particles (118,141) particularly montmorillonite clay, and surprisingly, retains infectious potential (118,213). Contaminated pastures have been shown to transmit disease (153,173) and faecal transmission via exposure to bedding of orally infected scrapie positive rodents, led to an infection rate of 80-100% among exposed animals confirming the infectious potential of animal excreta (208). Given the resistance of prions to environmental degradation and confirmation that infectious prions are shed in deer fecal excreta (229), and have been detected in freshwater of a CWD endemic area (163), it is likely that accumulation of these agents in the environment has lead to the infection of naïve animals. This is particularly relevant for 'herd ' animals such as wild cervids or agricultural animals. Combined with the evidence that PrP^{Sc} is present in urine (49,90,95,212), faeces (100,229), and saliva (147), the persistence and residence of prions in environmental reservoirs may be considerable (213).

1.2 Animal Models of TSEs

Animal models of TSEs have been instrumental in the discovery and detection of prions. Initially, animal infectivity models were the only way to verify and validate the infectivity and transmissibility of TSEs. As new TSE-

diagnostic methods emerge, animal models remain the gold standard for correlative verification between *in vitro*-based detection and *in vivo* infectivity. The subsequent development of transgenic animal models for various TSEs has significantly advanced the field of prion research by drastically condensing the latent period of disease onset. This has allowed considerably more data to be collected in a shorter time period as transgenic animals allow for disease progression within the lifespan of the host.

Due to complications with the species barrier, early studies in TSE research required transmission among the known natural hosts of a specific prion disease (*i.e.,* sheep for scrapie). Subsequent work in rodent models emerged, but the observed poor infectivity across the species barrier led researchers to believe that prion disorders were highly host-specific. It was soon realized however that the species-specific barrier could be overcome by serial infectivity passage of infected brain homogenates among syngeneic (*i.e.,* closely related) animals. For example, the well characterized 263K strain of scrapie in hamster models was originally isolated from sheep and through serial passage has been adapted to hamsters, and is no longer infectious to sheep (129). Since this time, various transgenic animal models for prions have been developed to overcome problems associated with transmissibility of prion diseases across species-specific barriers (33). Similar genetic manipulation technologies were used to create *prnp* knockout mice, whereby eliminating the prion protein would provide insights into this protein's physiological properties and to better understand its role in

infectivity and progression of disease (187). These advancements have aided in elucidating several characteristics unique to prion disease and diagnosis.

Animal bioassays are the most stringent test available, thus they are still used to validate all developed diagnostics. These assays are extremely time consuming and expensive, but necessary to ensure that new diagnostic tools are truly measuring the infectious agent of prion disease. Generally, two types of animal infectivity assays are used to determine infectivity potential; end point titration and incubation period. End point titration experiments use 10-fold serial dilutions of tissue or fluid homogenates (*i.e.*, brain, etc.) which are injected into animal cohorts. The lowest lethal dose killing 50% of animals (LD_{50}) is used to calculate infectivity units in the original inoculum (LD_{50} equals 1 infectivity unit) (113). Incubation period assays are viewed as a semi-quantitative method. Infectivity units are calculated based on a latent period model where death due to prion-induced disease is a function of time and dose in the original inoculum. This is usually inferred from laboratory dose response curves that are known to be fairly consistent within any given animal model, and are host, strain and tissue specific (190).

1.3 Diagnostics

Extensive research has focused on the development of diagnostic techniques to overcome the many challenges associated with diagnosis of prion diseases. Included in the specific challenges to diagnostics are: similarities in the structure and amino acid sequence of PrP^{C} and PrP^{Sc}, the extensive tissue distribution associated with some but not all prion diseases, the low level of

sensitivity necessary to measure infectious prions in tissue other than brain, and the necessity to correlate with animal bioassay infectivity. Indeed at least one example of infectivity in the absence of abnormal prion (*i.e.,* PrP^{Sc}) has been documented (137). For a diagnostic method to be useful it would need to successfully overcome most, if not all, of the challenges listed.

PrP^{Sc} is the only known disease-associated marker of TSEs. PrP^{Sc} has been shown to co-purify with infectivity (78), and *prnp* knockout ($prnp^{0/0}$) mice are resistant to prion disease (33). For this reason, most diagnostic approaches have focused on detection of PrP^{Sc} in host tissues. Due to the similarities between the normal and infectious prion protein, methods of detection have had to rely on the unique biophysical characteristics of PrP^{Sc}. Methods have focused on PrP^{Sc} resistance or partial resistance (149) to proteases, treatment with heat and/or acid, insolubility in chaotropic agents (*i.e.,* urea, guanidinium chloride) (55), conformational differences between isomers or a combination of these properties, typically followed by antibody detection. Several methods have been developed each having specific advantages and disadvantages (Table 1.5).

The necessity of ante-mortem diagnostic techniques capable of measuring low levels of infectious prions is becoming increasingly more apparent. The resistance, persistence, extreme infectious nature and transmission potential of prions makes them a serious threat to both human and animal populations. Human transmission of disease has been reported via insufficient disinfection of surgical instruments (25), human blood transfusion (142), organ transplantation (69) and hormone replacement therapy (47,183). Confirmation of the

involvement of both the secretory and excretory systems in some animal prion diseases poses additional challenges to the containment of these environmentally persistent and prevalent diseases (49,90,95,100,147,229).

At present, all Canadian Food Inspection Agency (CFIA) confirmatory diagnostic tests for prions are post-mortem (36). The only ante-mortem diagnostic tests utilized to date have focused on readily accessible tissues displaying PrP^{Sc} deposition. These diagnostic tools have to rely on intact tissues known to be infected with prions during the course of disease. Thus, they are limited by the distribution and extent of PrP^{Sc} in the tissue selected, and the stage of disease, along with the sensitivity of the detection method (*i.e.,* Western blot) used. Their universal applicability is also restricted due to the diverse tissue and fluid distribution of PrP^{Sc} and is dependent on the prion disease and species infected. Thus, these techniques have typically focused on animal prion diseases with known PrP^{Sc} tissue distribution (*i.e.*, scrapie and CWD lymphoid tissue). Successful ante-mortem immunohistochemistry (IHC) detection has been reported in the 3rd eyelid of sheep (165), the palatine tonsil of sheep (211,241) and deer (255), and the rectal mucosa of sheep (72,88,89) and elk (221). Although these are conceivably logical ante-mortem tests for some animal TSEs, they are time consuming, technically demanding, and may require sedation of the animal, making them somewhat impractical. The applicability of these techniques for pre-clinical diagnosis or confirmation at various time points is also limited as they focus on tissues that cannot be repeatedly sampled (*i.e.*, 3rd eyelid, tonsil). A review of potential ante-mortem diagnostic methods is provided in Table 1.6.

Table 1.5 Advantages and disadvantages of available diagnostic techniques for prion diseases.

	Advantages	Disadvantages
Histology	• Recognizes pathological hallmarks	• Confusion of similar pathologies associated with non-infectious TSE-like diseases • Invasive • Post-mortem tissues
IHC	• Combination of antibody and pathology	• Invasive • Post-mortem tissues (few ante-mortem tissues – *i.e.*, 3rd eyelid, tonsil)
WB	• Distinguish between different forms of disease (*i.e.*, strains) • Application to various tissues and fluids	• Time consuming • Labour intensive • Destroys PrP^{sen} (PrP^{sen} = PrP^{Sc} destroyed by PK digestion)
ELISA	• Detection improved over WB • Automated (*i.e.*, large sample screening)	• Detection limit cannot measure extremely low levels in all tissues/fluids • Destroys PrP^{sen}
CDI	• Measures both PrP^{sen} and PrP^{res} • No proteolytic degradation of sample	• Specific antibody requirements that recognize differences between PrP^{C} and PrP^{Sc}, these antibodies are not available for all species
PMCA	• Enhances detection by amplifying PrP^{Sc} to detectable levels • Applicable to buffy coat and a variety of fluids/tissues/species/matrices • Automated	• Requires a substantial normal brain homogenate supply (PrP^{C} from species of interest) • Difficult to get consistent reproducible results • Requires a detection method such as WB or ELISA (PK digestion)
EPA	• Adaptable to existing diagnostic platforms with reported lower detection levels	• Requires a detection method WB/ELISA after application. Downstream methods (WB/ELISA) destroys PrP^{sen}
iPCR	• Exponential amplification can be achieved utilizing established ELISA platforms • Versatility to various samples	• Necessity of complete PK digestion of PrP^{C} needs to be verified • Reliant on the exact specificity and sensitivity of detection antibody
Animal infectivity	• Gold standard of infectivity • Potential to explore cross species infectivity	• Time consuming, labour intensive and expensive • Complications with species barriers (multiple passages may be required)

Table 1.6 Evaluation of potential prion ante-mortem diagnostic tools.

	Ante-mortem potential	Limitations
IHC	Yes	Applicable only to bodily tissues with known PrP^{Sc} (cannot be applied to fluids)
WB/ELISA	?	Not sensitive enough alone, but can be utilized in combination with other techniques
EPA	Yes	Patented technology
iPCR	Yes	Utilizes upfront antibody-based detection which has limited sensitivity
Cell free conversion	No	Requires PrP^{Sc} in excess of PrP^{C} for conversion therefore does not reflect in vivo conditions
ACC	No	Lacks a measureable marker of infectivity (no PrP^{Sc} to detect)
rPMCA/QuIC	Yes	Infectivity of product not confirmed
PMCA	Yes	Requires numerous rounds/cycles to be highly sensitive

1.3.1 *Early diagnostics*

Historically the diagnosis of prion diseases has relied on histology (hematoxylin and eosin); the microscopic evaluation of brain tissue post-mortem for typical characteristic appearance of spongiform encephalopathy. Unfortunately, similar pathologies associated with non-infectious spongiform-like encephalopathies have been problematic for pathologists and resulted in confusion in confirming diagnosis.

Following the discovery of scrapie-associated fibrils (150) as primarily being composed of prion protein (*i.e.*, PrP) (37), antibody-based technologies emerged as the diagnostic choice for TSEs. Immunohistochemistry (IHC) became the gold standard for TSE diagnosis in the 1980s and is still used today for confirmatory purposes. Most antibodies unfortunately are unable to distinguish between the two isoforms of prion protein (PrP^{C} and PrP^{Sc}), thus current methods of IHC utilize formic acid and high temperature (autoclaving) to destroy PrP^{C} located in the tissue prior to immunostaining for PrP^{Sc} (81). It is still unclear what forms of PrP^{Sc} are being detected by IHC-based techniques (207). Both histology and IHC utilize only a small amount of tissue post-mortem and are completely reliant on the quality of the sections provided. Diagnosis can be difficult if the tissues are autolyzed, contain an insufficient amount of material, or if incorrect section of tissue is selected (102,256). Although IHC is still considered to be the gold standard in confirmation of prion diseases many factors need to be considered, including, the specificity of the antibody to the species being evaluated, the inefficiency or potential inability to diagnose preclinical animals, and the importance of adequate tissue sections and preparation.

Antibodies are used regularly in the detection of prions, but as previously mentioned, typically require the destruction of PrP^{C} in order to detect PrP^{Sc} as most monoclonal and polyclonal antibodies cannot distinguish between the two isomers (37). A list of some of the antibodies commonly used for the detection of prions can be found in Table 1.7. Of the antibodies listed several are very broad and useful in detection of PrP in numerous species, while others are only effective in one or a limited number of species. Knowledge of the prion epitopes (*i.e.*, amino acids [aa] sequence) recognized by various antibodies have been utilized in attempts to further understand the structural differences and conformational changes occurring during the conversion of PrP^{C} to PrP^{Sc} (70).

Recent advances have resulted in the development of a new class of antibodies capable of distinguishing PrP^{Sc} from PrP^{C}; these include OCD4, 15B3, and the YYR antibodies. The OCD4 antibody, is a monoclonal specific for DNA, or a DNA-associated molecule, in the case of PrP^{Sc} (266). The 15B3 monoclonal antibody specifically precipitates bovine, murine or human PrP^{Sc}, but not the normal conformer, suggesting that it recognizes an epitope common to PrP^{Sc} from different species (132). The YYR antibody, binds a tyrosine-tyrosine-arginine (YYR) repeat exposed as conformational changes to PrP occur (176). The use of this antibody must be exercised with caution, as there is the possibility that it may bind to other proteins in the test sample having a YYR repeat (92). The entire potential for this new class of antibodies has not yet been recognized and thus far they are not suitable for the direct identification of PrP^{Sc} in IHC (37), although their application in other diagnostics may be imminent (81).

Table 1.7 Antibodies used for detection of PrP.

Antibody	Specificity/cross reactivity	Epitope recognized	Reference
3F4	Human, baboon, hamster, cat (does not recognize any other mammalian species)	MKHV of murine aa 109-112	Kascsak *et al.*, 1987 (124)
6H4	Human, bovine, ovine, rabbit, mink, a variety of primates	DYEDRYYRE of bovine aa 144-152 of human	Sigurdson *et al.*, 1999 (216)
F89/160.1.5	Human, bovine, ovine, deer, elk	RPLIHFGSDYEDR aa 146-159 of bovine	O'Rourke *et al*., 1998 (166)
F99/97.6.1	Ovine, bovine, mule deer, elk, white-tailed deer	QYQRES aa 220-225 of bovine	Spraker *et al.*, 2002 (222)
Bar224	Ovine, cervid (does not recognize human, mouse)	aa 141-151 of human	Tamguney *et al.*, 2009 (229)
34C9	Bovine, rabbit, pig. mule deer (does not react with human, murine or hamster)	LIHFG of bovine aa 138-142 of human	Langeveld *et al.* 2006 (136)
4B4 (Scrapie)	Ovine, feline, rabbit, mule deer, elk (does not recognize other mammalian species)	Epitope site mapped appears non-linear	Vasan *et al.*, 2006 (242)
6D11	Human, bovine, ovine, mule deer, elk, mouse, hamster (not yet tested in other species)	Unknown	Pankiewicz *et al.*, 2006 (175)
7D9 (BSE)	Bovine, ovine, mule deer, elk, murine	Unknown	Murphy *et al.*, 2009 (161)
8H4	Bovine, ovine, hamster, chimpanzee, squirrel, deer, elk, various primates (reacts with recombinant prion protein or native PrP^{Sc})	aa 175-185 of murine	Zanusso *et al.*, 1998 (263)
3O8	Human, hamster, ovine (weak) and bovine (weak)	aa 106-126 of human	Vincent *et al.*, 2000 (244)
12F10	Human, bovine, ovine	aa 144-152 of human	Morel *et al.* 2004 (156)
P4	Ovine (does not react with BSE in ovine)	GGGGWGZGGSHSQWNK aa 89-104 of ovine	Stack *et al.*, 2004 (224) Langeveld *et al.* 2006 (136)
Bar233	Ovine, murine, human (hamster, bovine – unknown)	FGNDYEDRYYRE aa 145-156 of ovine aa 141-156 of human	Morel *et al.*, 2004 (156)

Table 1.7 (cont'd) Antibodies for detection of PrP.

Antibody	Specificity/cross reactivity	Eptiope recognized	Reference
R35	Deer, elk, ovine, bovine, murine, hamsters, possibly other species.	CGQGGTHGQWNKPSK aa 101-115 of hamster	Race *et al.*, 2002 (194)
R505	Cervid	SQWNKPSKPKTN aa 100-111 of cervid	Raymond *et al.*, 2000 (196)
SAF32	Human, hamster, bovine, ovine, murine	Octarepeat region located in the N-terminal region	Bolton *et al.*, 1982 (23) Prusiner *et al.*, 1997 (186) Prusiner *et al.*, 1998 (187) Marcotte and Eisenberg, 1999 (145)
SAF53	Human, hamster, murine (does not react with bovine, ovine)	DYEDRYYREN aa 144-153 of hamster	Morel *et al.* 2004 (156)
SAF54	Human, hamster, bovine, ovine, murine	aa 142-160 of human	Bolton *et al.*, 1982 (23) Prusiner *et al.*, 1997 (186) Prusiner *et al.*, 1998 (187) Marcotte and Eisenberg, 1999 (145)
SAF61	Hamster, murine, bovine, ovine, human	aa 142-160 of human	Mouillet-Richard *et al.* 2000 (157)
SAF70	Hamster, murine, bovine, ovine, human	aa 142-160 of human	Nishida *et al.* 2000 (164) Vincent *et al.* 2000 (244) Demart *et al.* 1999 (61)
SAF83	Hamster, murine (does not react with bovine, ovine, human)	Unknown	Bolton *et al.*, 1982 (23) Prusiner *et al.*, 1997 (186) Prusiner *et al.*, 1998 (187) Marcotte and Eisenberg, 1999 (145)
SAF84	Hamster, bovine, ovine, murine (does not react with human)	aa 160-170	Bolton *et al.*, 1982 (23) Prusiner *et al.*, 1997 (186) Prusiner *et al.*, 1998 (187) Marcotte and Eisenberg, 1999 (145)
Sha31	Bovine (used in BioRad TeSeE detection kit)	YEDRYYRE aa 145-152 of bovine	Morel *et al.* 2004 (156)

1.3.2 *Western blot*

The development of the Western blot (WB) assay, the most well characterized and validated screening protocol (113), has allowed for the measurement of the PrP^{Sc} in a much shorter time frame (1-2 days). This method uses a combination of protease digestion and heat degradation, coupled with gel electrophoresis and antibody detection. Protease digestion is routinely employed to distinguish between protease-resistant forms of the prion molecule (PrP^{Sc}) and protease-susceptible forms (PrP^{C}). Proteinase K (PK) treatment is used to hydrolyze PrP^{C} and to cleave the amino-terminus end of PrP^{Sc} resulting in protease resistant 27-30 kD core protein (approximately 142 amino acids in length) of the PrP^{Sc} molecule. PrP^{Sc} is then visualized post gel electrophoresis and antibody detection as three bands varying in molecular mass (di-, mono-, and unglycosylated). The intensity of the distinct banding pattern, can be quantified to determine the amount of infectious material present in the tissue analyzed, while also serving as a unique 'fingerprint' used to differentiate between prion disease forms (*i.e.*, strains).

Western blotting is time consuming, labour intensive, and has reduced sensitivity due to the destruction of potentially protease sensitive PrP^{Sc} (207). Its advantages include the direct quantification of protease resistant PrP^{Sc} and the ability to distinguish between different prion strains (*i.e.*, typical versus atypical scrapie in sheep) and different prion diseases within a single species (*i.e.*, scrapie versus BSE in sheep) (113). The detection limit of most WB applications has

been shown to be 10-20 pM (5-10 pg of PrP) using 263K scrapie infected hamster brain homogenates (113,139).

1.3.3 *Enzyme-linked immunosorbant assay*

Enzyme-linked immunosorbant assay (ELISA) platforms have also been developed for diagnostic purposes. Most ELISA-based platforms utilize PK digestion, followed by antigen capture of PrP^{Sc} through immobilization of primary antibodies onto a plastic well with subsequent secondary antibody detection via chemiluminescence. The detection limit of 2 pM (113) is improved over WB, and its large volume processing capacity makes it a valuable screening tool. ELISA-based screening platforms still require confirmation by WB.

A variation in the ELISA platform which does not depend on proteolytic degradation of PrP^{C} is the conformational-dependent immunoassay (CDI). CDI utilizes high affinity antibodies that recognize an exposed region (*i.e.*, epitope) of PrP^{Sc} (34,207). The ratios of PrP^{C} and PrP^{Sc} are then measured by a time-resolved fluorescence detection system, with the differences between the native and denatured forms of the test sample signifying the presence or absence of PrP^{Sc} (206). Since CDI does not rely on PK digestion it detects protease-sensitive PrP^{Sc}, estimated to comprise approximately 90% of the total PrP^{Sc} (207), potentially accounting for the increased sensitivity associated with this methodology compared to other ELISA-based platforms.

1.3.4 *Ultrasensitive tools*

Current regulatory (*i.e.*, CFIA) confirmatory diagnostics protocols (IHC, WB and ELISA) are not sensitive enough to accurately detect extremely low

levels of prions and therefore are only validated in brain and/or lymphoid tissue dependent on species and prion disease being tested (36). The development of ultrasensitive tools used alone or in conjunction with standard confirmatory diagnostics has been an intensely studied research area, especially since the discovery that prion diseases such as vCJD transmit to other individuals via blood (142). Early prognosis of prion diseases would aid considerably in the development of therapeutic agents, along with minimizing the exposure of naïve individuals. Described below are the most promising ultrasensitive tools that have come to the forefront in diagnostic research for enhanced detection of prions (summarized in Table 1.6).

1.3.4.1 Epitope Protection Assay

In the epitope protection assay (EPA), PrP^{C} and PrP^{Sc} are chemically reacted with a short-lived and highly-reactive chemical, peroxynitrous acid (ONOOH), which modifies selective amino acids in the monomeric PrP^{C} and exposed epitopes of PrP^{Sc}, but not the internal sequestered PrP^{Sc} epitopes in the aggregated form. After disaggregation with a standard chaotropic agent, such as guanidine hydrochloride (GdnHCl), the hidden sequestered epitopes of PrP^{Sc} are exposed and detection is accomplished by conventional immunoassays (140). This assay is made ultrasensitive with a combination of magnetic and fluorescent beads for detection of PrP^{Sc} in blood. Antibody combinations have been modified to adapt the assay to detect prions in multiple species (140). This method has successfully detected PrP^{Sc} spiked in human blood plasma at the femtogram level (171).

1.3.4.2 Immuno-Quantitative PCR

The second potential tool in sensitive detection of low level prions is the real time immuno-polymerase chain reaction (iPCR) technique (also immuno-quantitative PCR, immuno-quantitative real time PCR [iq-RT-PCR]). This technique is designed to directly detect prion proteins by ELISA and subsequently amplify a nucleic acid detector molecule, attached to the target antibody, for signal detection and quantification. The sensitivity of iPCR is limited by the specificity and sensitivity of the antibody to the target antigen (11). Lack of antibody specificity or incomplete digestion of PrP^C will lead to false positives, while a lack of antibody sensitivity may result in a lack of detection (*i.e.*, false negatives) (11,12). Initial attempts at utilizing iPCR led to 100% specificity with 10-fold increases in sensitivity over ELISA in cases of sporadic CJD (86,87). In other model systems a 1000-fold sensitivity over ELISA has been reported using scrapie–brain homogenate bound to polyvinylidene difluoride (PVDF) membranes (197) and a million fold increase in sensitivity has been reported along with consistent detection of recombinant PrP^C within hamster brain homogenates (12). These data imply that the sensitivity of the method may rely at least partially on the substrate utilized, but this does not discredit the possibility that a million fold increase in sensitivity over current screening diagnostics could allow for the detection in tissues other than brain.

1.3.4.3 *In vitro* prion propagation/amplification techniques

The task of recreating prions in a test tube is immensely challenging, but benefits from research in this area include better understanding the mechanisms of

prion disease and providing insight into physiological conditions affecting disease events. The first attempt of mimicking prion conversion via an *in vitro* assay was in 1992 (195,200) although attempts to convert mouse and hamster PrP expressed in cell culture failed. This trial revolutionized the thought process of prion researchers and lead to the subsequent development of several *in vitro* assays.

1.3.4.3.1 *Cell-free conversion assays*

The first successful *in vitro* 'cell-free conversion' assay was reported in 1994 (131). The cell free conversion assay utilized GdnHCl-treated PrP^{Sc} purified from prion-infected brains and radio-labeled PrP^{C} from mouse fibroblast cells to measure the conversion of PrP^{C} to PrP^{Sc}. The stoichiometry of the assay required that PrP^{Sc} needed to be in excess of PrP^{C}, (*i.e.,* 50 times more PrP^{Sc} than PrP^{C}) in order for a minimal amount of conversion to occur; approximately 10-20%, measured by autoradiography (200).

In 1999, Saborio *et al.* (205) attempted cell-free conversion using purified substrate (*i.e.,* purified PrP^{C} and PrP^{Sc}), but were unsuccessful. Conversion was only possible when cell components were not purified and remained in a cell lysate form (amount of amplification not quantified). This represented an improvement for *in vitro* cell-free conversion assays since PrP^{Sc} was required in only 10 times excess of PrP^{C} (205). This finding underscored the necessity of unidentified factors required for the conversion process.

Baskakov (16), in 2005 introduced an *in vitro* assay termed 'autocatalytic conversion' (ACC). ACC uniquely involves the re-folding of denatured recombinant PrP (rPrP) in the absence of PrP^{Sc} (15). Urea or GdnHCl are used to

denature rPrP, while continuous shaking induced the folding of the ß-oligomers into amyloid fibrils (15,17,22). The formation of amyloid fibrils can be monitored by fluorometry and the conversion rate depends on a number of parameters (*i.e.*, pH, concentrations of urea). The advantages of this assay include, the presence of a disulfide bond in the rPrP, which closely resembles the native state of prions, along with the complete elimination of both the cellular substrate (PrP^{C}) and infectious seed (PrP^{Sc}), making it an excellent system to study the aspects of spontaneous disease (200).

As is, these *in vitro* systems are currently not viable ultrasensitive diagnostic tools either because they require PrP^{Sc} in excess of PrP^{C} (cell-free conversion, cell lysate conversion assay) which is contradictory to the true prion disease scenario, or they have no PrP^{Sc} marker (ACC) for detection purposes. More importantly, none of these tools have been shown to conclusively amplify the infectious form of the prion protein.

1.3.4.3.2 *Protein Misfolding Cyclic Amplification*

The PMCA assay has emerged as a novel technology that uses PrP^{Sc} as a template for propagating the misfolding of native PrP^{C} from brain homogenates using a series of incubation and sonication cycles (40,42,218). The first essential requirement of PMCA is a source of normal PrP (*i.e.*, PrP^{C}), serving as the template to advance the conversion reaction. The PrP^{C} source is typically crude brain homogenates. It is critical that the source of normal brain homogenate be con-specific with the species from which the disease originates (*i.e.*, use of hamster brains as a PrP^{C} source for amplification of hamster 263K scrapie). In

some studies, PMCA has been used to address the species barrier by using brain homogenates from xeno-specific hosts (*i.e.*, different species) as the source of PrP^C (38). Difficulty in obtaining consistent and reproducible amplification in some prion disease models has been overcome with the use of transgenic mice as a source of PrP^C (134). The use of a crude brain homogenates emphasizes the fact that unidentified co-factors associated with brain tissues play an important role in template directed misfolding. The second requirement of PMCA is a 'seed' or 'infectious unit' to trigger the misfolding chain reaction. This can either be infected brain tissue spiked into the assay or infected tissues, fluids or environmental samples containing infectious prion particles. Thirdly, PMCA requires the incubation of the samples at a stable temperature of 37^oC (143). The constant temperature mimics the temperature that is maintained within most warm-blooded animals. Lastly, sonication is required for conventional PMCA. PMCA employs repetitive cycles of sonication and incubation. The purpose of sonication is to disrupt aggregates of PrP^{Sc} that form during incubation. Bursts of sonication break up aggregates or PrP^{Sc} seeds to provide additional 'seeds' that are able to recruit and convert more molecules of PrP^C. The PMCA assay also appears to require a minimum infectious seed that amplifies PrP^{Sc} without a reduction in infectivity, and for which the size of PrP^{Sc} aggregates that are generated correlate with animal infectivity (21,182,250).

PMCA became the first *in vitro* assay capable of efficient conversion of cellular prion with PrP^{Sc} substrate in limiting quantities, exploiting the finding that unidentified factors were required for prion conversion and propagation

(205). The PMCA method is extremely sensitive and has been used to successfully detect prions in the buffy coat layer of blood collected directly from the heart of a scrapie-infected hamster at an advanced stage of disease (42). The most significant advantage of the PMCA assay over other assays, is its level of sensitivity, able to amplify ~26 molecules of protein monomers (*i.e.*, 1×10^{-12} dilution) of scrapie hamster brain to detectable levels by WB after 7 rounds of PMCA (203). Moreover, it is the only *in vitro* assay that has been confirmed to generate *infectious* prion units (40).

The original PMCA assay involved the use of individual sonication probes as a form of manual sonication , and achieved >50x amplification after only 5 cycles (204). By 2005 amplification rates between 20-100x of infectious product were routinely obtained by Castilla *et al.* (40). This level of amplification was quickly surpassed with the development of serial automated PMCA (saPMCA) capable of generating 12 $\log_{10}$ of amplification in 7 days (203). The automated sonicator system offered two key improvements over its manual predecessor: elimination of cross-contamination potential between samples and higher throughput of samples (41), both essential in the successful establishment of an ultrasensitive diagnostic tool.

Success in amplification of PrP^{Sc} by conventional serial PMCA lead to modification attempts by other laboratories. Supattapone *et al.* (226) developed a non-denaturing protocol which was believed to more closely mimic an *in vivo* system because of concerns that detergent (*i.e.*, sodium dodecyl sulfate) and sonication could be degrading essential cellular protein factors and/or altering

normal biochemical reactions, thereby not replicating PrP^{Sc} as it would in a true biological system (200). Unfortunately the use of non-denaturing conditions led to significantly less amplification (only ~10x amplification) (226). Therefore the usefulness of PMCA under non-denaturing conditions has been restricted to the identification of cellular co-factors involved in the PrP^{Sc} conversion. Lucassen *et al.* (143) and Deleault *et al.* (58), respectively, have demonstrated that RNA molecules (but not DNA) and accessory anions may be necessary for PrP^{Sc} amplification. Enhanced amplification of PrP^{Sc} in the presence of poly A RNA (58), has since been implicated in *de novo* generation of PrP^{Sc} (*i.e.,* the generation of protease resistant protein previously recognized as PrP^{Sc} in the absence of a PrP^{Sc} seed) (237).

Caughey and colleagues (6,7), have developed two techniques based on modifications to conventional PMCA. The first, recombinant PMCA (rPMCA), as the name suggests, utilizes recombinant PrP (rPrP) prepared from transformed *E.coli*, allowing for amplification of $rPrP^{Sc}$ which is distinguishable from spontaneously formed rPrP-res due to self-aggregation. The rPMCA assay was successfully applied to detect PrP^{Sc} in cerebral spinal fluid (CSF) of animals at the terminal stage of disease, however the increase of PrP^{Sc} was not quantified for the experiment (6). The second method, termed 'quaking-induced conversion' or 'QuIC', replaced the sonication steps of rPMCA with automated tube shaking to achieve similar, yet more reproducible levels of conversion than rPMCA (200). The QuIC methodology has been applied to sheep brain derived PrP^{Sc}, and was able to distinguish between infected and uninfected sheep via amplification and

detection of PrP^{Sc} in CSF in less than 24 hours (7). These modifications to conventional PMCA use a more reliable and available PrP substrate, report greater simplicity and ease of duplication, however, as of yet the proof of an infectious PrP^{Sc} conversion product has not been reported in the literature. Although non-denaturing PMCA, rPMCA and QuIC provide their own unique advantages in the study of prions they have been unable to obtain the same level of amplification as conventional PMCA, nor provide proof of amplification of an infectious product.

The PMCA assay has been applied to detection of prion disease in a variety of brain samples, both with experimental and natural TSEs of humans and animals (134,159,220). There is some uncertainty as to whether all prions strains can be amplified *in vitro* (227). PMCA has been successfully used to detect prions in the brains of pre-symptomatic scrapie infected hamsters (220), and recently for detection of PrP^{Sc} in soil (213), urine (90,159) and faeces (100,133). It is also currently being used to address species barriers (38,39,90). The development and optimization of PMCA as an ultrasensitive tool capable of detecting *infectious* prions in a multitude of matrices which accurately reflects the pathobiological process of disease progression (*i.e.*, template directed propagation) has been essential in understanding the basic biology of TSE diseases. The applicability of PMCA is apt to accelerate the development of clinical intervention (*i.e.*, prophylactic drugs) and control strategies (*i.e.*, disinfection technologies) for both human and animal prion diseases.

As mentioned previously, the PMCA assay appears to require a co-factor associated with brain tissues for the conversion of PrP^{C} and the consequential propagation of PrP^{Sc}. Prior to the elucidation of the prion hypothesis, it had always been speculated that conversion of PrP^{C} to PrP^{Sc} could not possibly occur in the complete absence of DNA or RNA (3). Prusiner and colleagues postulated the 'protein-X' theory (73,233), based on experiments where mice expressing only human PrP (HPrP) or mice expressing both mouse PrP and mouse-human chimeric PrP (MHu2M) were susceptible to human prion disease, whereas mice expressing both human and mouse PrPs were resistant to human prion disease. This indicated mouse PrP^{C} inhibited transmission of human prions but had little effect on the conversion of mouse-human chimeric PrP. These findings were interpreted as mouse PrP^{C} binding more preferentially to rodent protein X than to human PrP^{C}, thus inhibiting the conversion of human PrP^{C} into PrP^{Sc} (219).

Although the protein X theory was the first true line of evidence of an additional protein (which may or may not be a protein) required in PrP^{C} to PrP^{Sc} conversion, it was further complemented with evidence of decreased conversion in all *in vitro* assay systems using purified or partially purified components (122,205). Various laboratories still continue to investigate the exact nature of this yet to be identified co-factor (or co-factors) with numerous potential candidates possible (73). Under 'non-denaturing PMCA' conditions, specific RNA molecules (but not DNA) and/or synthetic accessory polyanions (*i.e.*, poly A, poly dT, heparin sulfate proteoglycan) might act as scaffolds or surfaces that facilitate PrP^{C} and PrP^{Sc} interaction (57,58,60). Research into this area may

eventually elucidate the true co-factor or co-factors associated with prion conversion. This finding would be extremely instrumental in the development of targeted vaccines for prevention of prion disease or therapeutic agents for those already affected by these neurological disorders.

For PMCA to be useful in ante-mortem diagnostics, it must be applicable to an easily accessible, abundant tissue or bodily fluid. The most convenient and logical bodily fluid, at least from a human perspective, would be blood. In the 1990's, animals were used to demonstrate that prion infectivity was associated with blood components during both the incubation period and clinical phases of disease (26,28,29,232). Not until 2005 did Castilla *et al.* (42) present the first biochemical detection of PrP^{Sc} (in buffy coat of terminally ill hamsters) with PMCA. Pre-symptomatic detection of PrP^{Sc} in hamster blood (*i.e.*, buffy coat) by PMCA was identified shortly thereafter (202). Based on the distribution of infectious prions in animal and human (CJD) diseases it is likely that PrP^{Sc} is circulating in symptomatic and possibly pre-symptomatic individuals, but at a much lower level than can be detected in the brain. Currently confidence in human blood supplies in treating or managing life-threatening conditions is in question without a reliable diagnostic tool to confirm the absence of infectious prions in donors.

Without the incorporation of PMCA current *in vitro* diagnostic techniques have been unable to detect the presence of PrP^{Sc} (infectivity) known to be associated with blood. This in part could be a consequence of the estimated 100-1000x lower PrP^{Sc} levels than in the brain (93). However, the application of

PMCA to blood and its components is not without other challenges. The presence of small amounts of plasma or serum have been reported by to inhibit the PMCA reaction, as well as interfere with Proteinase K (PK) digestion (41,230). Due to the inhibition effects of components of plasma, research has tended to focus on buffy coat or leukocyte preparations (42,202,237). Detection in plasma has been limited to only a portion of the positive samples (Sc237 hamsters) tested in one study (159) and detection with a novel technology (surround optical fiber immunoassay [SOFIA]) that does not require proteolytic degradation of the sample prior to detection after limited-serial PMCA and immunoprecipitation in a second study (199).

Minimal scientific literature about the specific nature of plasma inhibition to *in vitro* amplification techniques (*i.e.*, PMCA) or diagnostics is available. This is due to both the lack of understanding of what components in blood fractions, including plasma, are imparting a negative effect on amplification or detection and/or if the level of PrP^{Sc} is simply too low for post amplification detection techniques. Recently, however, the masking of PrP epitopes in the presence of plasma components, specifically low density lipoproteins (LDLs), which impaired antibody detection, was observed (10). Evidence has also confirmed that various blood preparation protocols have a significant effect on PrP^{C} content measured in blood plasma (262). Both findings are similar to problems encountered with application of ultrasensitive diagnostics to urine, where outer membrane proteins and/or pre-concentration attempts complicated or inhibited PrP^{Sc} detection (77,90,159). Although the progress associated with detection of prion diseases in

blood matrices has quickly advanced from confirmed infectivity, just over 10 years ago, to successful application of an ultrasensitive diagnostic (see Figure 1.1), the development of an ante-mortem diagnostic for this fluid has yet to be announced.

1.4 Research hypothesis and objectives

An overview of the significant contributions in the development and modification of PMCA by the major laboratories over the past decade have been compiled in Figure 1.2. From the timeline, it is interesting to note that a considerably long lag time exists between publication of the original description of PMCA to implementation in other laboratories, implying that replication and optimization of PMCA under ideal (brain) conditions, let alone in bodily fluids or other matrices, may take serious time and effort.

An ultrasensitive diagnostic test such as PMCA, that is able to consistently and reproducibility detect low level prions and which mimics the pathobiological processes *in vivo*, is essential for better understanding the biology, diagnosis and treatment of prion diseases. The PMCA protocol is currently the most ultrasensitive tool for amplification and detection of low level *infectious* prions within bodily tissues and fluids. The adaption of the PMCA protocol to detection of infectious prions in plasma would truly provide a significant advancement in the ability to collect and process samples for ante-mortem diagnostic purposes. The adaptation of PMCA to plasma may also aid in understanding the species specificity of prion diseases and conceivably shed light on some of the misconceptions currently associated with a lack of detection in blood (plasma)

and/or the impact of blood-brain barrier interactions in the establishment and propagation of prion disease.

The working hypothesis of this thesis is that infectious prions circulate in the plasma of pre-symptomatic and clinically ill 263K scrapie-infected hamsters and ultrasensitive tools such as PMCA can be adapted to detect misfolded prions in plasma. In order to prove or disprove the hypothesis, two research objectives needed to be addressed. First, a standardized approach to the PMCA assay was developed where the specific requirements of PMCA were manipulated in order to understand the impact of modification or variation of the key parameters of the assay on the degree of amplification achieved. The second objective was to adapt the optimized PMCA assay for detection of 263K hamster prions in plasma, and subsequently apply in trial applications to clinically-ill 263K hamster plasma samples (with matched controls). The results of this thesis lay a framework for adoption of the PMCA assay for detection of prions in plasma and opens new and exciting opportunities to understand the pathobiology of these unique diseases.

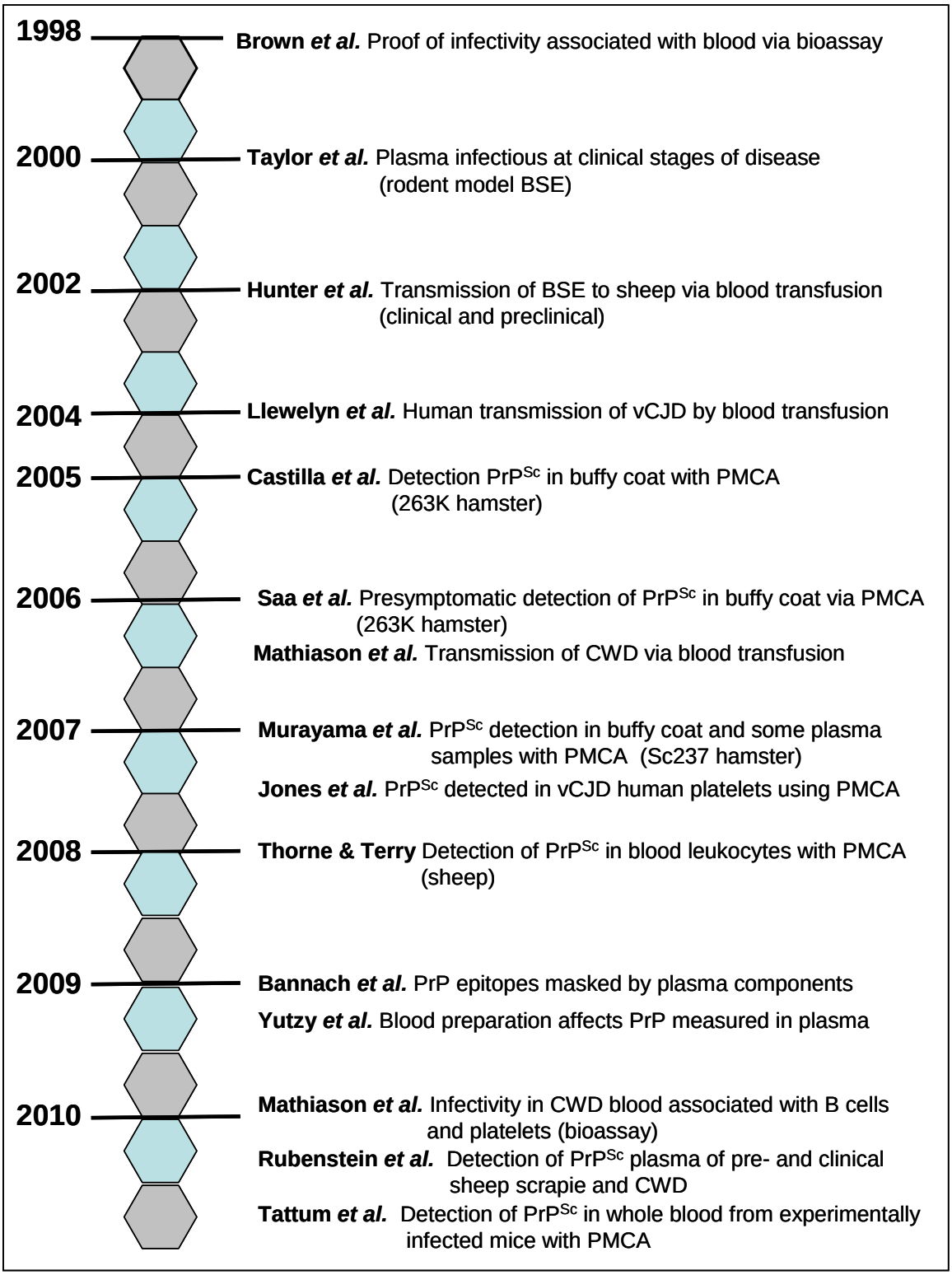

Figure 1.1 Timeline of key developments associated with detection and characterization of infectivity of prions associated with blood components.

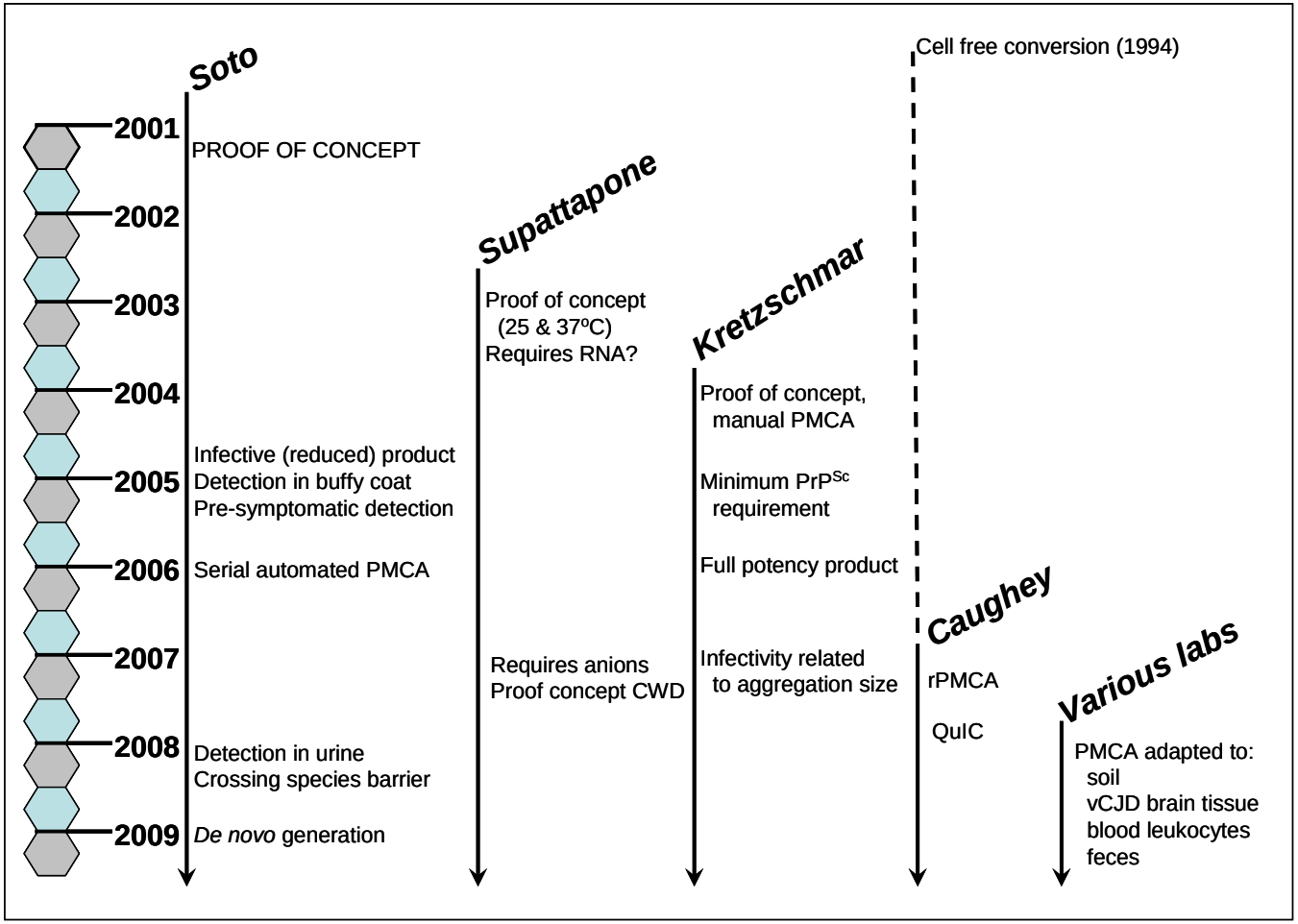

Figure 1.2 Timeline illustrating key developments with the PMCA assay.

2 MATERIALS AND METHODS

2.1 Conversion buffer solutions and preparation

2.1.1 *Conversion buffer reagents*

Phosphate buffered saline (PBS) was prepared as 1X PBS, pH 7.4 according to Castilla *et al.* (41). The exact desired concentrations of the specific chemicals, along with supplier and catalogue number are detailed in Table 2.1. A 5M NaCl solution was prepared for use in the conversion buffer (CB) (Fluka/Sigma-Aldrich, Toronto, ON, catalogue #71376). The salt solution was discarded six months after preparation date. Triton X-100 (Calbiochem, San Diego, California, USA, catalogue #648466) solution was diluted to a final concentration of 20% in 1X PBS (Table 2.1). The 20% Triton X solution was prepared at least one day before CB preparation to allow the detergent to completely mix with the PBS. Initially Triton X-100 solution was used undiluted, however after experiencing difficulty in accurately pipetting the small amounts required, the dilution adjustment was made to the protocol. The dilution modification of Triton X-100 was particularly critical in experiments where detergent concentrations were investigated. Ethylenediaminetetraacetic acid (EDTA) 0.5M was purchased from Gibco (Invitrogen Canada Inc., Burlington, ON, catalogue #15575-038) and used directly in the CB preparation. A Complete Protease Inhibitor Cocktail was purchased from Roche Diagnostics (Laval, QC, catalogue #1836145) and added according to manufacturer's instructions (*i.e.*, 1 tablet/50 mL volume of 1X PBS).

Table 2.1 Phosphate buffered saline preparation, including specific chemicals, suppliers and catalogue numbers.

Chemical	Supplier	Catalogue #	Final concentration
NaCl	Fluka	71376	137 mM
KCl	MPBiomedicals	R19668	2.7 mM
Na_2HPO_4	Calbiochem	567547	10 mM
KH_2PO_4	Calbiochem	529568	1.8 mM

2.1.2 *Conversion buffer preparation*

The CB was prepared according to Castilla *et al.*, 2006 (41) with the addition of 5 mM of EDTA as described by Castilla *et al*., 2004 (37). Solutions and components were added to 1X PBS (see preparation above) in the order and concentration listed in Table 2.2 and mixed well to completely dissolve the Complete Protease Inhibitor prior to use. CB was ideally prepared at least 4-6 hours in advance of use.

Table 2.2 Conversion buffer preparation.

Component	Final concentration
NaCl	0.15 M
EDTA	5 mM
Trition X-100	1%
Complete Protease Inhibitor Cocktail	1X

2.2 Animal handling protocols and tissue preparation

All hamster handling and tissue preparation protocols used in this thesis adhered to the Canadian Council of Animal Care (CCAC-Canada) guidelines. Protocols were approved through onsite Animal Care Committees at either the Canadian Food Inspection Agency – Ottawa Laboratory Fallowfield (CFIA-OLF) or the University of Alberta.

2.2.1 *Normal hamster brains*

Normal hamster brains were prepared from four to six week old female Syrian Gold hamsters (Charles River Laboratories International, Inc., Wilmington, MA, USA). Hamsters were sacrificed shortly after delivery by exposure to excess carbon dioxide (dry ice in a kill box). Reflexes and breathing were measured to confirm hamsters had expired prior to further processing. Upon confirmed death, each hamster was pinned by its extremities to a polystyrene (Styrofoam) board. Scissors were used to cut the skin of the abdomen laterally across the body of the hamster and then centrally up to the heart making every effort not to disturb/puncture any organs. Excess skin was pinned back and a large (20 gauge) needle with a piece of rubber tubing between the needle and syringe (50 mL volume) was immediately inserted in the left ventricle and clamped in place. Cold PBS with 5 mM EDTA was perfused throughout the hamster circulatory system using the syringe. After the 30-50 mLs of PBS/EDTA solution had been flushed through the hamster circulatory system, the syringe, clamp and pins were removed. The hamster was then flipped over, with backside up, forceps were used to tightly grasp the nose of the hamster and scissors were used to cut and sever the spinal cord. Additional cuts were made on either side of the head from the severed spinal cord towards each ear so that the skin could be flipped forward to expose the cranium. While maintaining a tight hold on the hamster nose with the forceps, scissors were used to cut from the spinal cord opening through the jaw bones near the bottom of the skull. A lateral cut was then made between the eyes to detach the front of the skull from the brain. One

final cut through the centre of the skull bone on the top of the brain was made so that the sides of the skull could be easily flipped away to expose the entire brain. A well perfused brain was completely white, lacking any pink or red staining associated with blood hemorrhage. The brain was then gently pulled away from the skull. Each hemisphere, along with as much of the obex as possible, was placed in a small snap cap tube (1.5 mL Eppendorf, Mississauga, ON, catalogue # 2236 334-4). Tubes containing brain were then either placed on dry ice or immediately in the freezer at -80^{o}C. Normal hamster brains were shipped on dry ice to the CFIA-OLF facility and stored at -80^{o}C until required.

2.2.2 *Infected hamster brains*

Three to four week old female Syrian Gold hamsters (Charles River Laboratories International, Inc., Wilmington, MA, USA) were exposed orally (100 μL) or by intraperitoneal (IP) injection (50 μL) to an inoculum of scrapie positive brain homogenate upon receipt at the CFIA-OLF Animal Facility. The positive scrapie 263K inoculum consisted of a 10% pooled brain homogenate in PBS with ampicillin. All infected animals were confirmed positive by both ELISA and IHC.

Syrian Gold hamsters displaying clinical signs of scrapie, typically 95-110 days post inoculation (dpi), were euthanized with carbon dioxide and the brains harvested in as short of time as possible. Perfusion was not performed on infected hamster brains. Half the brain was preserved in formalin and the other half frozen in an inventory repository (-80^{o}C). A macroscopic necropsy of the abdominal

cavity was completed to rule out any other disease process. For the purposes of PMCA only frozen inventory was utilized.

2.3 Preparation of brain homogenates for PMCA

2.3.1 *Normal Brain Homogenate (NBH)*

Perfused Syrian Gold hamster brains were removed from -80^{o}C and allowed to thaw on ice. An appropriate weight was weighed in small disposable petri dish for the preparation of a 10% NBH. Typically 1 g +/- 0.2 g of normal (non-infected) hamster brain was placed in the Potter homogenizer (VWR International, Mississauga, ON) with 9 mL of CB, and manually disrupted with 15-20 strokes in the homogenizer. The mixture was then transferred (poured off) to a 15 mL centrifuge tube and placed in the refrigerator (4^{o}C) or left on ice for at least 30 min. The homogenate was centrifuged at 950 x *g* (Eppendorf 5804R, Eppendorf Canada**,** Mississauga, ON) for 40 sec and the clarified supernatant used for experimentation. The pellet was discarded along with the zone between clarified supernatant and pellet. The low centrifugal speed/force used to clarify the NBH preparation was important to ensure that essential co-factors associated with the brain and required for conversion in the PMCA reaction were not removed.

2.3.2 *Infectious Brain Homogenate (IBH)*

Brains of infected 263K Syrian Gold hamsters were supplied by CFIA-OLF. Infected brains were not perfused when the hamsters were sacrificed and therefore infectious brains were not free of blood. After sacrifice, infected scrapie brains were stored at -80^{o}C until needed for homogenate preparation. A 10% IBH

in CB was prepared in disposable 1.5 mL ribolyzer tubes with zirconium beads (BioRad, Hercules, CA, USA) using the FastPrep homogenizer (Qbiogene Inc., Carlsbad, CA, USA) at a setting of 4 (equivalent to 4.0 m/sec), for 20 sec, followed by a 30 min incubation at 4^{o}C, and centrifugation (Eppendorf 5804R, Eppendorf Canada**,** Mississauga, ON) at 950 x *g* for 40 sec. The supernatant was then removed and the pellet discarded ensuring that the zone between supernatant and pellet was not removed or disrupted. The IBH was then aliquoted into smaller volumes, to avoid issues related to repeated freeze/thaw cycles, and stored at -80^{o}C for future use.

2.4 Hamster plasma

2.4.1 *Collection of hamster plasma*

Blood from normal and scrapie infected Syrian Gold hamsters was collected at the CFIA-OLF. Scrapie infected hamster plasma was collected from animals that had progressed to the point of displaying typical clinical signs of disease (typically 95-110 dpi). The blood samples were collected via transcardial puncture from anesthetized animals using an S-Monovette blood collection system (Kent Scientific Corporation, Torrington, CT) into a syringe with anticoagulant (K_2EDTA). After blood collection the hamster was euthanized with carbon dioxide. Non-infected (*i.e.*, negative) blood samples were collected in the same manner from purchased negative control animals (Charles River Laboratories International, Inc., Wilmington, MA, USA) that were received at CFIA-OLF a day or two prior to sacrifice. All blood samples collected were

stored at 4°C until plasma separation was completed. Normal hamster plasma was also purchased from Innovative Research (Southfield, MI, USA).

2.4.2 *Separation of hamster plasma*

After hamster blood collection, plasma was extracted as soon as possible (with storage at 4°C until processed). Blood tubes were inverted several times to remix the components, followed by a 25 min centrifugation (Thermo Scientific IEC3000) at 1400 x *g*. The top layer, consisting of plasma, was carefully extracted using a sterile transfer pipette and stored at -80°C.

2.5 PMCA equipment

2.5.1 *Sonicator 3000MXP*

A Misonix Sonicator Model 3000MXP (Misonix Inc Farmingdale, NY, USA), was used for the work described in this thesis. This make and model of sonicator was used in the initial PMCA work described by Castilla *et al.* (40), and is used by most research groups (6,42,134,202). The sonicator 3000MXP consists of a microplate cup horn and a generator. The cup horn offers indirect sonication and functions as a high intensity water bath. The generator is the power source for the cup horn and is programmable, allowing the user to preset the duration of individual bursts of sonication, incubation time between cycles of sonication, and the number of cycles to be completed (*i.e.*, total sonication time). The system was attached to power supply via a battery back-up system during all experimentation to ensure consistency of performance in the event of power outages or surges.

The microplate holder simply consisted of a piece of plexiglass with appropriately sized and organized holes for the 0.2 mL PCR tubes. The holder

sits on top of the microplate horn edge, above the water level of the reservoir, and allows the bottom of the PCR tubes (*i.e.,* the sample) to be submersed in the water bath at an appropriate (and consistent) distance from the horn. This microplate holder also allowed samples to be placed in various configurations (*i.e.,* an appropriate distance from each other) for maximum efficiency and ease of documentation of sample position for reference and/or organization.

The sonicator 3000MXP microplate cup horn was housed within the acoustic enclosure provided with the instrument. This acoustic enclosure was placed inside a bench top incubator. In order to achieve an approximate temperature of 37^{o}C in the water reservoir, the temperature setting of the bench top incubator was adjusted accordingly. In this case, an incubator temperature setting of ~33^{o}C, resulted in a temperature of ~37^{o}C or slightly below (1-2^{o}C) of the water in the cup horn. A temperature at or slightly below 37^{o}C was preferable since the temperature of the water in the bath increased as more sonication bursts were completed.

Typical sonication cycles consisted of 40 sec of sonication followed by 29 min 20 sec of incubation for a duration of 16 hours (32 cycles). Each PMCA round was initiated with a 40 sec sonication burst (unless otherwise specified) and samples were retrieved within 15 mins of the last sonication burst for consistency. Power setting 6 (or 60%) was used unless otherwise specified.

2.5.2 *Water bath*

A temperature controlled water bath (Fisher Scientific, Ottawa, ON, catalogue number: 15462S2) set to 37^{o}C was used for incubation only (37^{o}C)

controls. A makeshift circulating water bath was designed to determine the effect of incubation temperature on PMCA efficiency. Specifically, a variable flow peristaltic pump (VWR International, Missisauga, ON, catalogue #138762) was used to pump water from a temperature controlled water bath (Fisher Scientific, Ottawa, ON) to the water reservoir of the PMCA horn and back. Both the water bath temperature and pump flow were adjusted to maintain an approximate temperature of 35-37^{o}C in the reservoir where PMCA samples were sonicated. When trials with the makeshift circulating water bath were performed the Sonicator 3000MPX was not housed within the incubator, but on the laboratory bench.

2.6 PMCA experimental set-up

Using the prepared hamster NBH as substrate and hamster IBH as seed, PMCA experiments were initiated. A dilution series was prepared in 1.5 mL snap-cap microcentrifuge tubes (Eppendorf, Mississauga, ON), and typically consisted of several dilutions of the initial 10% IBH in the 10% NBH stock (Table 2.3). Once all tubes in the dilution series had been prepared they were mixed by inverting (never vortexed) in an effort to avoid destroying important components/co-factors. Eighty µL of each dilution in the series (except the 1:10 dilution) was then aliquoted into three labeled 0.2 mL thin walled PCR tubes with attached-caps (Nalgene/Nunc International, Rochester, NY, USA,catalogue #250875). All three sets of tubes were frozen at -80^{o}C for at least 20 min. Two sets of the dilution series were removed and allowed to thaw at room temperature. The remaining frozen sample remained at -80^{o}C for the duration of the

experiment and was considered the time zero/frozen control. One set of the two thawed series was placed in the 37^{o}C water bath, and any amplification achieved in this series was attributed to temperature alone (*i.e.*, no sonication). The final series was exposed to sonication that is, placed in the water reservoir of the PMCA sonicator housed in the incubator. Multiple replicates for the variables tested were from independent experiments. An independent experiment refers to an experiment where the CB was prepared fresh, a previously unused normal brain was homogenized in the fresh CB, and the dilution series was set-up as outlined below (Table 2.3). Each independent experiment was carried out on the same PMCA machine, but at different times or on different days. The only variable that remained unchanged in independent experiments was the IBH used.

Table 2.3 Dilution series preparation for PMCA experiment set-up.

Dilution of IBH[a]	10% NBH (µL)	10% IBH (µL)	Total volume (µL)	% IBH
0 (NBH only)	1250	0	1250	0
1:10	180	20	200	1
1:100	1125	125 of 1:10	1250	0.1
1:500	1000	250 of 1:100	1250	0.02
1:2500	1000	250 of 1:500	1250	0.004
1:12500	1000	250 of 1:2500	1250	0.0008
1:62500	1000	250 of 1:12500	1250	0.00016
1:312500	1000	250 of 1:62500	1250	0.000032
1:1562500	1000	250 of 1:312500	1250	0.0000064
1:7812500	1000	250 of 1:1562500	1250	0.00000125
1:39062500	1000	250 of 1:7812500	1250	0.000000256

[a] refers to dilution of the initial 10% IBH

2.7 PMCA optimization, standardization and development

While developing and standardizing the PMCA protocol, various modifications were made to the basic experimental design in order to optimize the

assay. An illustrated flow chart of the basic experimental design is provided in Figure 2.1 for reference. The basic PMCA protocol was used to maintain consistency of all parameters of PMCA while varying only a single parameter during the course of optimization. The parameters that were examined during optimization of PMCA were divided into external or internal variables. In addition to these variables the use of serial PMCA (sPMCA) was investigated.

2.7.1 *External variables*

2.7.1.1 Duration

Modifications to the time/duration of the samples exposed to PMCA sonication was accomplished by simply removing the required samples from the water reservoir at specified time intervals (*i.e.*, 3, 6, 24, 48 and 68/72 hours).

2.7.1.2 Pre-incubation of samples in sonicator

In some instances, samples were exposed to a pre-incubation period (30 mins) at 37^{o}C prior to the first cycle of sonication by placing the samples in the water reservoir of the sonicator and the PMCA generator was not initiated until the specified pre-incubation period had lapsed. All other samples were exposed to their first burst of sonication as soon as placed into the cup horn water reservoir.

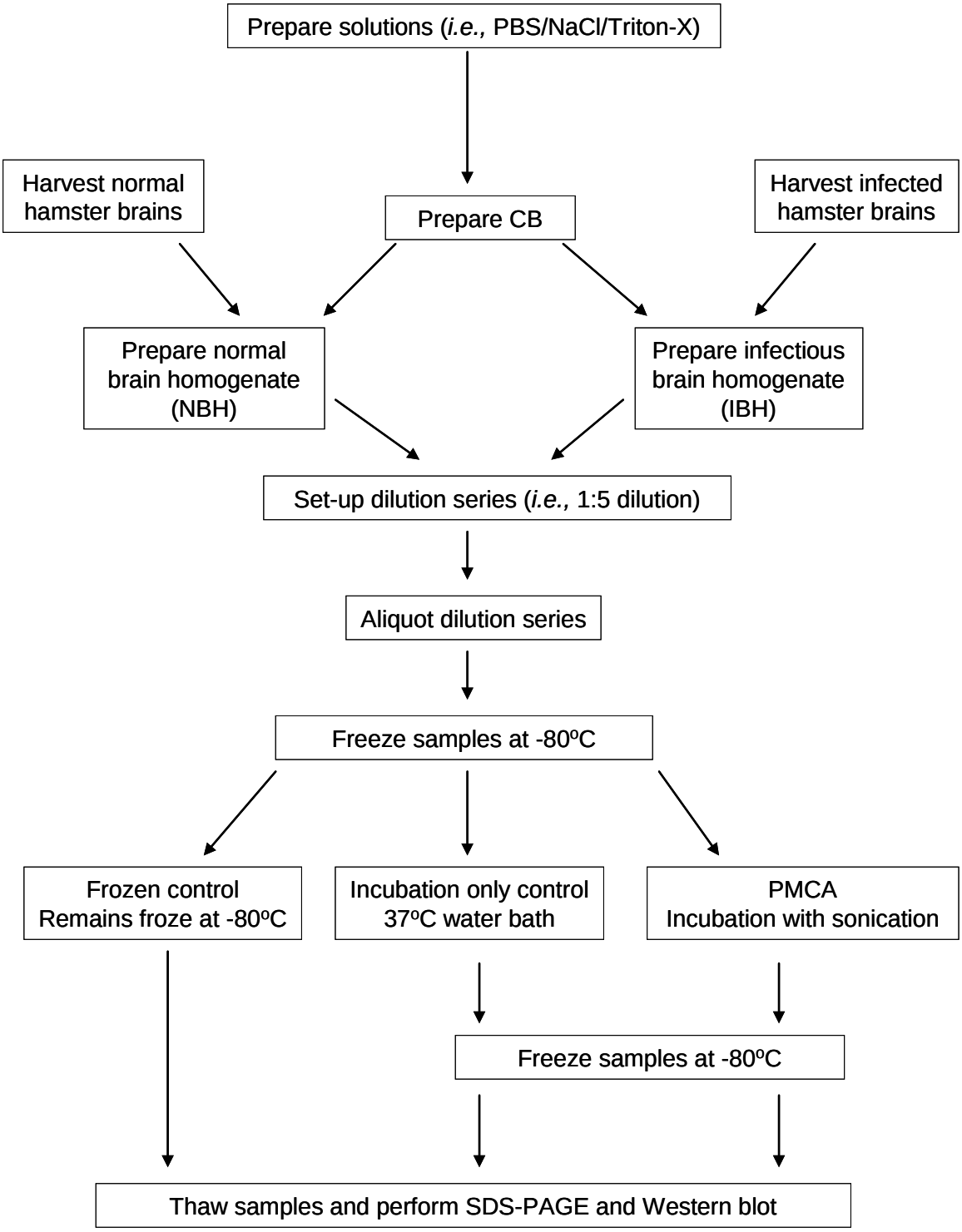

Figure 2.1 Overview of PMCA experimental process, from solution preparation and experimental set-up to sample processing and analysis.

2.7.1.3 Requirement of sonication and intensity of sonication

One set of experiments was carried out by incubating an additional series of samples in a water bath set to 37^{o}C (similar to the water temperature set in PMCA sonicator). Sonication intensity was adjusted from setting 4 to 10 (*i.e.*, 40%-100%) by re-programming the generator of the Misonix 3000MPX to determine optimal results.

2.7.1.4 Temperature

A peristaltic pump with water bath system (as described above) was used to vary temperature in the system (35.5-40^{o}C).

2.7.1.5 Sample vessels

Work by Atarashi *et al.* (6), suggested that 200 µL 'attached-cap' thin walled PCR tubes should be used for PMCA assays (as opposed to 'strip-cap' tubes). Both attached-cap (Nalgene/Nunc International, Rochester, NY, USA, catalogue #250875) and strip-cap tubes (Nalgene/Nunc International, catalogue #248161) were compared during PMCA optimization in the present thesis.

2.7.2 *Internal variables*

2.7.2.1 Storage effects associated with conversion buffer (CB)

The CB preparation was carried out as described above. Freshly prepared CB (*i.e.*, 'fresh' CB) was prepared on the day of experimental set-up compared to 'old' CB that was obtained from a previously prepared batch of CB ($\geq$1 week) stored at 4^{o}C.

2.7.2.2 Detergent concentration

An NBH solution with a final concentration of 1.1% Triton X-100 was prepared. From this NBH stock, two further dilutions were made in NBH (without detergent) to achieve final concentrations of 1.0% and 0.9% Triton X in NBH.

2.7.2.3 Effects of freezing samples prior to PMCA

In these experiments a dilution series was kept at 4^{o}C (or on ice) until amplified by PMCA and compared to samples frozen at -80^{o}C prior to PMCA.

2.7.2.4 Perfused vs non-perfused normal hamster brains

NBH was prepared from perfused or non-perfused hamster brains to determine the effect of residual blood within preparations. Previous data suggested that contamination of preparations with blood components could lead to inhibition of PMCA (41).

2.7.2.5 Effects of freeze-thaw cycles on IBH for seeding PMCA

Small aliquots of IBH were frozen in an -80^{o}C freezer for 10 min then allowed to thaw at room temperature for at least 10 min prior to re-freezing. This was repeated for 1, 10, or 25 times and samples subsequently used to seed the PMCA assay.

2.7.3 *Serial PMCA (sPMCA)*

Additional rounds of PMCA (or sPMCA) were carried out to determine the dynamic range of the assay upon multiple rounds of PMCA. The PMCA products from the previous round were diluted 1:5, 1:10, 1:100 or 1:1000 into new NBH (*i.e.*, fresh) preparation and exposed to the next round of PMCA.

2.8 Application of PMCA to detection of PrP^{Sc} in hamster plasma

Upon optimization of the PMCA assay, experiments were carried out to determine if the optimized PMCA assay could be adapted to detection of PrP^{Sc} in blood from infected hamsters. Details of the individual experiments investigating the adaptation and application of the PMCA assay to hamster plasma are described below.

2.8.1 *Dynamic range of sensitivity of optimized PMCA assay in presence of plasma*

Non-infectious or normal plasma, hereafter referred to as 'normal plasma', was purchased from Innovative Research (Southfield, MI, USA). An initial concentration of 10% (final volume) of Syrian Gold hamster plasma with K_2EDTA as the anticoagulant (catalogue #IHM-N-06) was used in the preparation of CB which was used to prepare hamster NBH. The control NBH (*i.e.*, 0% plasma) was diluted with additional PBS (10% final volume) to compensate for the dilution of all other CB and NBH components. The dilution series using IBH were prepared as usual and the conditions of PMCA carried out on samples with and without normal plasma present.

2.8.2 *Species specificity of PMCA plasma amplification*

Xenospecific plasma (*i.e.*, not hamster) was also purchased from Innovative Research, Southfield, MI, USA from bovine (catalogue #IBV-N-06), ovine (catalogue #ISH-N-06) and murine (catalogue #IMS-N-06) sources and used in 10% final volume concentrations to prepare hamster NBH as described above. The addition of xenospecific plasma to the PMCA assay was carried out to test for possible host-specific effects of plasma on the species barrier associated

with *in vitro* amplification. The four NBH with plasma preparations (*i.e.*, bovine, ovine, murine and hamster) were then used to set-up the usual dilution series spiked with hamster IBH and subjected to one round of PMCA.

2.8.3 *Assessing the inhibitory effect of plasma concentrations*

Normal plasma was added at concentrations of 5, 10, 25, or 50% total volume in CB used to prepare NBH. Additional round(s) of PMCA were applied as detailed in sPMCA section with and without dilution of the previous PMCA product in NBH with plasma. Serial PMCA was performed with and without the presence of contiguous plasma. Contiguous plasma refers to maintaining a specified concentration (5 or 10%) of plasma in the NBH diluent of the subsequent rounds of PMCA.

2.8.4 *Application of the PMCA assay for prion detection in plasma samples from scrapie infected 263K hamsters*

Plasma samples separated from whole blood collected by CFIA-OLF (see collection of hamster plasma for exact details) were added in a 1:10 dilution to hamster NBH without an infectious seed. Serial PMCA was carried out for multiple rounds (6 or 7 rounds) with the previous round PMCA product diluted into NBH with (10% plasma) and without (0% plasma) contiguous plasma present. Then SDS-PAGE and WB were performed on the PMCA products from each round.

2.9 SDS-PAGE

Proteinase K (PK) was carried out on 263K hamster experimental samples with a final concentration of 200 µg PK/mL. Samples included, but were not limited to: -80^{o}C frozen samples, incubation only samples, and PMCA products.

Specifically, the 20 mg/mL PK included in the TeSeE Sheep/Goat purification kit (BioRad, Hercules, CA, catalogue #355-1165) was diluted 1:20 in Milli-Q® water (1 mg PK/mL). Ten uL of the 1 mg PK/mL solution was added to 40 µL of experimental sample (total volume 50 µL resulting in a final concentration of 200 µg PK/mL), vortexed and incubated at 37^{o}C +/- 2 ^{o}C for 20 min to digest all PrP^{C} in the sample. An equivalent volume of 2x Laemmli buffer solution (*i.e.,* 50 µL) was added to reduce and denature the samples. Samples were then vortexed and incubated at 100^{o}C +/- 5 ^{o}C for 5 min. Laemmli buffer (2x) solution was prepared according to BioRad TeSeE Purification and Detection kit; specifically, 28.5 mL of Laemmli sample buffer (BioRad, Hercules, CA, catalogue #161-0737), 0.6 g sodium dodecyl sulfate (SDS, Sigma-Aldrich Canada Ltd, Oakville, ON, catalogue #L4390-100G) and 1.5 mL ß-mercapto-ethanol (Sigma-Aldrich Canada Ltd, Oakville, ON, catalogue #M-7154). If Western blotting (WB) was not immediately performed, samples were refrigerated overnight for processing the next day or frozen at -20^{o}C for longer storage.

Precast Criterion XT 12% Bis-Tris polyacrylamide gels (BioRad, Hercules, CA, USA, catalogue #345-0118) were placed in the migration tank (Criterion XT Cell, BioRad, Hercules, CA, catalogue #165-6001) and the Criterion XT cell was filled with migration buffer. Migration buffer was prepared according to manufacturer's specifications (XT-MOPS, BioRad, Hercules, CA, catalogue #161-0788). The comb of the gel was carefully removed from each gel and storage buffer rinsed from each of the gel wells with migration buffer just prior to sample loading.

Samples were heated to 100^{o}C +/- 5^{o}C for 4 min prior to loading. Equal sample volumes (30 µL) were loaded into all wells, along with one lane of Kaleidoscope prestained standard (BioRad, Hercules, CA, USA, catalogue #161-0324) and one lane for molecular weight ladder (Magic Marker XP Western Standard, Invitrogen, catalogue #LC5602). Kaleidoscope standard was used to verify proper transfer from the gel to the membrane and the molecular weight ladder to identify the approximate molecular weights of the proteins. The Criterion XT cell power supply, HC Power Pac (BioRad, Hercules, CA, catalogue #164-5052), ran for 50 min at 200V.

2.10 Western blotting

Transfer buffer was prepared to a final concentration of 1X Tris/CAPS (BioRad, Hercules, CA, USA catalogue #161-0778 [10x stock]) and 15% absolute ethanol (Commercial Alcohols, Brampton, ON, Canada) in distilled water. Prepared transfer buffer was used within 48 hours of preparation. Polyvinylidene difluoride membranes (PVDF, BioRad, Hercules, CA, USA, catalogue #162-0177) were used that appropriately matched the gel dimensions. To condition the membrane, it was first immersed in 95% ethanol for 15 sec, then rinsed for 5 min in distilled water and allowed to acclimate for 10 min in prepared transfer buffer.

The SDS-PAGE (polyacrylamide gel electrophoresis) gels were carefully removed from between the plastic plates and placed in transfer buffer to equilibrate for at least 10 min prior to the transfer step. Assembly of the transfer cassettes was carried out according to the manufacturer's protocol. Disposable filter papers (BioRad, Hercules, CA, USA, catalogue #170-4085) and fiber pads

were soaked in transfer buffer to minimize/eliminate the potential for air bubble intrusion during the transfer. A gel roller (BioRad, Hercules, CA, USA, catalogue #165-1279) was used to ensure no air bubbles that may have formed between the layers remained. The Trans-Blot cell (BioRad, Hercules, CA, USA, catalogue #170-3946) was plugged into Power Pac HC (BioRad, Hercules, CA, catalogue #164-5052) and ran for 60 min at 115V.

After transfer the PVDF membrane was quickly removed and immersed in 1X PBS, then placed in 95% ethanol for 10 sec before rinsing for at least 5 min in distilled water. The membrane was incubated for a minimum of 30 min (room temperature, *i.e.*, 18-30^{o}C) with medium agitation using a platform rocker/shaker (Gel Surfer, Model GS1216), or overnight without agitation (*i.e.*, 4^{o}C), in 5% skim milk (Carnation, Nestle USA Inc, Glendale, CA) to block unoccupied membrane binding sites and prevent nonspecific binding of detection antibodies. The blocking solution was removed and the membranes were incubated at room temperature, under medium agitation using a rocker, with primary antibody 3F4 (Millipore, Billerica, MA, USA, catalogue #mAb1562). The 3F4 antibody binds hamster, human, cat, and baboon prion protein, and is not known to react with any other mammalian species. The 3F4 antibody was diluted to a final concentration of 0.0001 mg/mL (1:20,000 dilution of the 2 mg/mL stock solution) in PBS with Tween (*i.e.*, PBST). The PBST was prepared according to BioRad specifications with a final concentration 0.1% Tween-20 (BioRad, Hercules, CA, USA, catalogue #170-6531) in 1X PBS (BioRad, Hercules, CA, USA, catalogue #161-0780). After 30 min of incubation with primary antibody, the antibody solution

was poured off and membrane washed 3x with PBST. Two washes of 5 min and one wash of 10 min respectively. In certain experiments (*i.e.,* samples containing hamster plasma), SAF32 or SAF84 (Cayman Chemical, Ann Arbor, MI, USA, catalogue #189720 and #189775, respectively) were used as the primary antibodies for prion detection.

The conjugated secondary antibody, goat anti-mouse horse-radish peroxidase (HRP, BioRad, Hercules, CA, USA, catalogue #170-6516), was added to bind to the primary antibody. The membrane was immersed and agitated in secondary antibody, diluted 1:5000 in PBST, for at least 20 min. The membrane was washed with three washes (5, 10, 10 min respectively) and then rinsed with excess volume of 1X PBS to eliminate as much detergent (*i.e.,* Tween) as possible. To validate the specificity of secondary antibody binding, the primary antibody step was eliminated in some experiments.

The membrane was then removed from the PBS wash and allowed to drain on absorbent paper and placed in a plastic folder. Chemiluminescent detection was performed using the ECL reagent (Amersham, GE Life Sciences, Canada, catalogue #RPN2109) according to manufacturer's instructions. Excess ECL reagent and air bubbles were eliminated from the plastic folder using absorbent paper. With the membrane enclosed in the plastic folder it was placed in the dark for 5 min and then exposed using the Gel Doc XR System (BioRad, Hercules, CA, USA, catalogue #170-8170). The chemiluminescent signal was detected using manual adjustments with optimal exposure times, typically 3 exposures at 5, 10 and 15 min. The Gel Doc instrumentation and Quantity One software package

were used to label gel images. All gel images were then exported as jpeg images for comparison, analysis and result compilation.

2.11 Analysis of PMCA results

Where possible, gel images were used to calculate the amplification achieved with PMCA. This was accomplished by visually comparing the WB pattern (dilution series) of the -80^{o}C frozen controls to the post-PMCA (PMCA) dilution series. An increase or decrease in amplification was approximated by counting the number of dilutions where a PrP^{Sc} band was observed and using this number as the exponent to the base dilution factor, in this case 5. For example, if 3 dilutions in the series displayed bands after PMCA, and the final dilution yielded a band of equal visual intensity to those observed in the -80^{o}C frozen control, the calculated amplification rate would be 5^3, *i.e.*, 5*5*5 = 125x. If the post-PMCA product was greater in band intensity than the frozen control (-80^{o}C), the result was assigned a 'greater than' (*i.e.*, '>') value. If the band intensity in PMCA samples was weaker than the frozen control it was given a 'less than' (*i.e.*, '<') value. If amplification was observed in the lowest dilution of the series, this dilution with a 'greater than' value was used for calculation purposes. For average amplification calculation absolute values of amplification were used and the result rounded to 1 significant figure. In experiments where sPMCA was employed, the dilution between rounds of PMCA was included in the calculation of average amplification. For instance if 2 rounds of PMCA were performed with a 1:10 dilution between rounds the resulting average amplification was multiplied by the dilution factor (*i.e.*, 10).

3 OPTIMIZATION AND STANDARDIZATION OF THE PROTEIN MISFOLDING CYCLIC AMPLIFICATION ASSAY

3.1 Introduction

Protein Misfolding Cyclic Amplification (PMCA) was first reported as an *in vitro* method for the amplification of infectious prions in 2001 (204). The reproducibility, sensitivity and specificity (*i.e.*, strain amplification) of the PMCA assay has been described in several publications, the most notable of which detailed the ultra-efficient replication of infectious prions by automated PMCA (203). When this thesis was initiated (2006), relatively few laboratories were successful in replicating the amplification rates or consistency of the PMCA assay reported by Soto and colleagues.

Proof of the concept of PMCA and its' capacity to amplify prions was first established using a manual sonicator (Sonoplus 2070, Bandelin, Germany) (204), however other laboratories were unable to achieve similar levels of amplification. Saborio *et al.* (204) initially applied 5 pulses x 1 sec at power setting 40% once every hour to achieve 57.9 +/- 19.9 fold amplification after only 5 PMCA cycles. Lucassen *et al.* (143) demonstrated minimal amplification (10x) was possible with incubation at 37^{o}C for 16 hours in the complete absence of sonication. Bieschke *et al.* (21) were only able to consistently achieve 10x amplification with manual PMCA after 23 hours. Increased amplification (~300x), in their laboratory, could only be achieved with serial dilution of the PMCA product after every 10 cycles of PMCA into fresh NBH up to 10 rounds (21). This was further improved upon to amplification rates of 200,000x after 15 rounds of serial PMCA utilizing the manual PMCA system (182). Clearly discrepancies in amplification using the

manual PMCA protocol were apparent as shown in Table 3.1 (publications where degree of PMCA amplification [in $\log_{10}$] could be estimated based on data/results presented). The relatively few publications, and lag times associated with use of manual PMCA (Figure 1.2) implied that considerable standardization and optimization was required within each laboratory utilizing the assay.

The development of an automated PMCA (aPMCA) sonicator enhanced the potential of PMCA as an ultrasensitive diagnostic tool that could be readily adapted and standardized in various laboratories. The aPMCA offered three key improvements over its' manual predecessor: 1) elimination of cross-contamination potential between samples, 2) higher throughput of samples and 3) standardization of the assay (41). The introduction of the aPMCA also made it possible for the inventors to clearly validate the reproducibility, specificity and sensitivity of the assay described in detail below (203). Saa *et al*. (203) were able to demonstrate reproducibility based on sample position in the sonicator among different experiments, along with the influence of different, but equivalent infectious inocula (IBH) and different brain substrates (NBH). Minimal variation (not significant) was dependent on sample placement that was attributed to experimental variability rather than a positional effect. No statistically significant differences were seen between samples started on 7 different days where the first day experiment used a fresh NBH preparation, while the 6 additional experiments used NBH that was frozen prior to PMCA. The consistency between fresh and frozen NBH preparation results emphasized the utility of using either preparation as an NBH substrate, noting however that brain substrates must be frozen in

aliquots to avoid repetitive freeze-thaws. While addressing the use of 5 different, yet equivalent IBH inocula, all cases resulted in a large degree of amplification. Amplification was also produced in the use of 5 different hamster NBH substrates with one common inoculum. Evaluation by densitometric analysis revealed that variation of IBH inocula and NBH substrate resulted in 1 of 5 samples displaying a statistically significant lower level of amplification. These discrepancies were proven not to be associated with variation in the level of PrP^{Sc} or PrP^{C} in the samples, but believed to be due to individual variability among animals in PrP expression and/or conversion co-factors.

The specificity and sensitivity associated with the aPMCA also made it an attractive tool for diagnostic applications. Specifically Saa *et al*, (203) reported 100% specificity with PMCA based on a blinded study of 10 positive samples, along with 11 negative controls. Only with numerous rounds of PMCA (*i.e.*, >10 rounds) was a PrP^{Sc}-like signal ever detected in the absence of a PrP^{Sc} seed. Unfortunately the PrP^{Sc} detected after numerous rounds could not be disregarded confidently as a result of cross-contamination, nor confirmed as *de novo* generation. Lastly, the sensitivity of the PMCA assay was evaluated in relation to standard WB. Remarkably 3 rounds (100 cycles/round) of sPMCA was shown to be 3 billion times more sensitive than standard WB, which corresponds to >4000x more sensitive than animal bioassay.

The establishment of aPMCA assay greatly improved the potential utilization of PMCA as an ultrasensitive diagnostic tool, along with its application to further understanding the molecular basis of prion propagation (203). The

aPMCA assay also allotted for a platform that would potentially decrease inter- and intra-laboratory variation. However, after a thorough review of both the manual and automated PMCA publications from 2001 to 2007 and quantifying (or providing enough data to semi-quantify, *i.e.*, estimate) the degree of amplification in these manuscripts, it was clear that discrepancies still existed (Table 3.1). Interestingly, in a review of all publications utilizing PMCA in 2007 (10 in total), five were from (or in collaboration with) laboratories who previously published using the PMCA assay (58,82,134,198,251), one was from a laboratory familiar with earlier *in vitro* amplification techniques (6), and two were from Murayama *et al.* (159,160), a Japanese group that had previously established a variation of the aPMCA protocol utilizing a fully automatic cross-ultrasonic protein activating apparatus (ELESTEIN 070-GOT, Elekon Science Corp., Chiba, Japan), distinct from the Misonix automated sonicator. The two remaining publications from Jones *et al.* (120) and Seidel *et al.* (213), displayed the applicability of aPMCA to vCJD brain material and recovery of 263K brain homogenate in soils after extended periods of time, respectively. The appearance of only 2 out of 10 publications relating to PMCA independent of previous experience with the assay again exemplified the importance of optimization and standardization upon initialization of the PMCA assay in this thesis.

Numerous critical variables to the PMCA assay had been reported; ultrasound strength and frequency (220), pH (143), use of crude brain homogenates rather than purified homogenates (226), freeze-thaw effects on brain homogenates (which may decrease infectivity) (21), minimum ‘seed’ requirement

for amplification to occur initially (146) or upon serial dilution (182), and the necessity of accessory polyanion addition (58). However, no study had addressed the differences in procedures and protocols between laboratories to determine to what extent individual variables influenced the degree of amplification achieved. When comparing the methods for the aPMCA assay, small variations in preparation of NBH, CB components, PMCA samples tubes, duration of PMCA, sonication intensity, number of cycles and whether to start a PMCA reaction with incubation or immediate sonication, along with temperature were observed in the literature (Table 3.2 and Table 3.3).

The technical papers by Castilla *et al.*, 2004 (37) and 2006 (41), along with Saa *et al.* (201), were given careful consideration as they clearly specified the details of the PMCA procedure, along with helpful recommendations and troubleshooting techniques. Atarashi *et al.* (6) provided a detailed description of the rPMCA methodology to ensure reproducibility. Based on these select publications and the observed discrepancies in procedures between laboratories (Table 3.2) several key variables to the PMCA assay were identified and manipulated to determine the degree of impact on achievable amplification. Given the variability in published approaches to PMCA, this chapter focused on development and optimization of PMCA through systematically altering those variables perceived to affect PMCA.

Table 3.1 Comparison of PMCA publications presented in the literature from 2001-2007.

Laboratory Reference	Sonciator/ Assay	Amplification rate (fold), # PMCA cycles/time	Detectable PrP^{Sc}	NBH substrate/ Application	Estimated log amplification[a]
Soto 2001 Saborio *et al.* (204)	Manual/ PMCA	57.9 +/- 19.9 fold, 5 cycles	~6-12 pg	263K hamster BH	>1 $\log_{10}$ (5 cycles)
Supattapone 2003 Lucassen *et al.* (143)	Incubation only	>10 fold, 16 hours		Sc237 hamster BH	>1 $\log_{10}$ (16 hours of incubation at 37°C only)
Kretzshmar 2004 Bieschke *et al.* (21)	Manual/ PMCA	5 fold, 10 cycles, ~10 fold, 23 cycles		263K hamster BH	~1 $\log_{10}$ (23 cycles)
	Manual/ serial PMCA	~300 fold, 100 cycles		263K hamster BH	> 2 $\log_{10}$ (10 rounds = 100 cycles)
Soto 2005 Castilla *et al.* (42)	Automated/ PMCA	6600 fold, 140 cycles		263K hamster BH	>3 $\log_{10}$ (140 cycles)
	Automated/ serial PMCA	10 million fold, 214 cycles		263K hamster BH	7 $\log_{10}$ (2 rounds = 214 cycles)
Kretzshmar 2005 Piening *et al.* (182)	Manual/ PMCA	200000 fold, 150 cycles		263K hamster BH	>5 $\log_{10}$ (15 rounds = 150 cycles)
Soto 2006 Saa *et al.* (202)	Automated/ serial PMCA	10^{-12} fold		263K hamster BH	12 $\log_{10}$ (7 rounds = 1000 cycles)
Caughey 2007 Atarashi *et al*. (6)	Automated rPMCA		>=10 fg, 24 cycles	rHaPrP	~3 $\log_{10}$ (24 cycles)[b]
	Automated/ serial rPMCA		~50 ag, 48 cycles	rHaPrP	~ 6 $\log_{10}$ (2 rounds = 48 cycles)[b]
Independent 2007 Jones *et al.* (120)	Automated/ PMCA	1000 fold, 48 cycles		vCJD BH	3 $\log_{10}$ (48 cycles)
Supattapone 2007 Kurt *et al. (134)*	Automated/ PMCA	~6-27 fold, 96 cycles with deer NBH >200 fold, 96 cycles with Tg_{cer} NBH		Deer and Tg_{cer} BH	>2 $\log_{10}$ (96 cycles with Tg_{cer} NBH)

[a] estimated from data presented

[b] based on comparison to WB with detection of ~5-10 pg of PrP^{Sc}

Table 3.2 Comparison of PMCA assay methods (2004-2007).

	Castilla *et al.*, 2004 (37)	**Saa *et al.*, 2005 (201)**	**Castilla *et al.*, 2006 (41)**	**CEA (2007)**	**Kurt *et al.*, 2007 (134)**	**Atarashi *et al.*, 2007 (6)**	**BASIC PROTOCOL**
Tissue perfusion	PBS + 5 mM EDTA			No perfusion	PBS + 5 mM EDTA		
NBH preparation Homgenizer	Bandelin Sonoplus HD 2070[1]		Eurostar PWR BSC S1[2] or manual Potter homogenizer[3]	BioRad Ribolyser or manual Potter homogenizer	FastPrep[4] (setting 6.5 for 45 sec)	Not specified	Manual Potter homogenizer
Centrifugation of NBH (g/time)	2000 g 10 sec	5000 g 30 sec	2000 g 30 sec	1000 g 20 sec	2000 g 1 min	2000 g 2 min	950 g 40 sec
Storage of NBH	-80°C	-80°C	-80°C	-30°C	-70°C	-80°C	-80°C
PMCA sample vessel	0.2 mL thin walled PCR tubes					Nalgene/Nunc 248161[5] (0.2 mL PCR tubes)	
Sonicator	Misonix 2020[6]		Misonix S3000MXP[6]				
Sample holder			Adaptor rack	Floating 96 well plate	Not specified	Not specified	Adaptor rack
Volume in tubes	80 - 100 µL	80 µL	60 – 100 µL	50 – 150 µL	60 µL	80 µL	80 µL
Water in reservoir			140 mL	300 mL	160 mL	Half full	~140 mL
Duration/cycles	<100 hours	Not specified	24 hours/ 48 cycles	Varied	48 hours/ 96 cycles	24 hours/ 48 cycles	24 hours/ 48 cycles
Pre-incubation	1 hour	1 hour	30-60 min	30 - 120 min	Not specified	Not specified	30 min
Sonication/ Incubation cycles	40 sec 30 min	20 sec 1 hour	20 sec 30-60 min	Varied	20 sec 30 min	40 sec 1 hour	40 sec 30 min
Sonication intensity	40-80%	100%	80%	Varied	70%	100%	80%
Temperature/ Incubation	Shaking water bath between manual sonication pulses		S3000MXP in bench top incubator			Circulating water bath attached to reservoir of S3000MXP	S3000MXP in bench top incubator

[1]Bandelin Electronic, Germany
[2]IKA, USA
[3]VWR International, Edmonton, AB, Canada
[4]Qbiogene Inc., Carlsbad, CA, USA
[5]Rochester, NY, USA
[6]Farmingdale, NY, USA

Table 3.3 Comparison of conversion buffer preparation used with aPMCA.

	Castilla *et al.*, 2004 (37)	**Saa** *et al.*, 2005 (201)	**Castilla** *et al.*, 2006 (41)	**Atarashi** *et al.*, 2007 (6)	**Kurt** *et al.*, 2007 (134)	**BASIC PROTOCOL**
PBS	NaCl 8 g KCl 0.2 g Na_2HPO_4 1.44g KH_2PO_4 0.24g to a final volume of 1 L in H_2O pH 7.4				No PBS specified	See Castilla *et al.*, 2006 (41)
Conversion buffer	Prepared in PBS with the following components:					
NaCl	0.15 M					
Triton-X 100	1%			0.5%	1%	1%
Complete protease inhibitor cocktail	1X Complete protease inhibitor (Roche[a], #1836145)			1X EDTA free (Roche, #118361 70001)	1X (Roche, #1836145)	1X Complete protease inhibitor (Roche, #1836145)
EDTA	4 mM	5 mM	None	1 mM	None	5 mM
SDS	None	None	May include low concentration	0.05%	None	None
pH	7-7.3			7.4	Not specified	7-7.3
Additional components				recombinant PrP	Saponin 0.05% (Sigma)	

[a] Laval, QC, Canada

3.2 Results and Discussion

3.2.1 *Detection of prion protein by Western blotting*

Western blotting (WB) with 3F4 antibody was used for the detection of PrP^{Sc} throughout this thesis and for optimization of the PMCA assay (42,202-204). The monoclonal antibody, 3F4, detects both the normal and infectious form of the prion protein (PrP^{C} and PrP^{Sc}) dependent on the use of proteinase K (PK) digestion. Destruction of the normal cellular form of the protein (PrP^{C}) using PK digestion (200 µg/mL of PK for 20 min) allowed for the effective detection and quantification of PrP^{Sc} in samples. The prion protein displayed a higher

molecular weight (33-35 kD) in the absence of PK digestion which shifted to 27-30 kD for PrP^{Sc} after digestion (Figure 3.1). Typical of the 263K hamster species, the diglycosylated band of PrP was often the only band visualized after immunodetection (203,220). The absence of a banding pattern in the 'NBH only' lane of the WB confirmed complete destruction of PrP^{C} (appropriate PK concentration) and that the NBH substrate was completely void of detectable PrP^{Sc}. Hamster IBH samples (pre-PMCA) prepared at a dilution of 1:100 were routinely detected in WB, and for this reason it was chosen as the comparative control for estimation of the level of amplification achieved during optimization of the PMCA assay (Figure 3.1). For simplicity, these samples are referred to in this thesis as '-80^{o}C controls'.

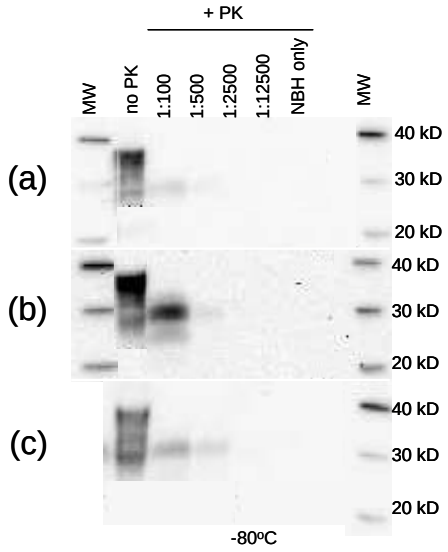

Figure 3.1 Western blot demonstrating the effectiveness of the monoclonal primary antibody 3F4 for detection of PrP^{Sc}. Replicate (a), (b) and (c) were from independent experiments. Infectious brain homogenate (IBH) was serially diluted (1:100-1:12500) into normal brain homogentate (NBH) and treated with (+PK) and without (no PK) Proteinase K (200 µg/mL) digestion. Samples were -80^{o}C frozen controls. Lanes labeled 'MW' represents the molecular weight markers at 40, 30, 20 kD.

3.2.2 *Basic PMCA protocol*

Of fundamental importance to this thesis was the selection of a basic PMCA method from which optimization of the assay could be performed. The technical publication of Castilla *et al.* (41) from 2006, clearly outlined aPMCA and its' parameters, and served as the primary source for this protocol (the most recent publication associated with the Soto laboratory at the time of initialization of this thesis work). Two deviations from this protocol were incorporated: 1) centrifugation (speed and time) of the NBH and 2) the addition of EDTA to the CB. These changes were to err on the side of caution based on recommendations that high centrifugal force might remove important membrane components necessary for PrP^{C} to PrP^{Sc} conversion (201), along with the successful reports of amplification with EDTA in the CB preparation by both Castilla *et al*. (37,42) and Saa *et al.* (201). This method is referred to as the 'basic protocol' for future reference in this thesis (see Table 3.2 and 3.3).

Using this basic 24 hour (48 cycles) PMCA protocol, PrP^{Sc} was reproducibly amplified *in vitro* by approximately 5x (Figure 3.2). Unfortunately this level of amplification was lower than all reports in the literature using either the manual or automated sonicators (21,42,143,203,204). The basic protocol, however, provided consistent reproducibility of results that served as the reference point for subsequently optimizing the conditions of PMCA.

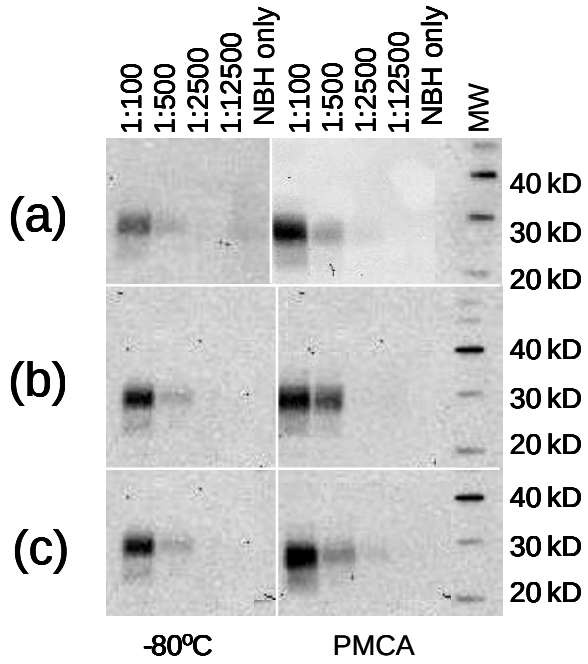

Figure 3.2 Western blot demonstrating *in vitro* amplification of PrP^{Sc} using the basic PMCA protocol (24 hours, 48 cycles). Replicate (a), (b) and (c) were from independent experiments. Infectious brain homogenate (IBH) was serially diluted (1:100-1:12500) into normal brain homogentate (NBH) and treated with Proteinase K (PK). Lane labeled 'MW' represents the molecular weight markers at 40, 30, 20 kD.

3.2.3 *Optimization and characterization of key variables influencing the PMCA assay*

With the basic PMCA protocol providing reproducible results with minimal amplification (*i.e.*, ≤5x), variables affecting the PMCA assay could be further investigated. Since many of the factors affecting the amplification efficiency of the PMCA reaction were not well understood or documented, it was important in the optimization process to characterize the key variables influencing PMCA. Variables investigated were determined from recommendations outlined in technical publications of the PMCA methodology by Castilla *et al.* (37,41) and

Saa *et al.* (201), along with variations in protocols displayed when comparing and contrasting methodologies (Table 3.2 and 3.3). For evaluation purposes, the variables investigated were divided into parameters that were external or internal to the sample itself; 'external' referring to variables that may have an indirect impact on the sample, such as sonication power, and temperature, etc., whereas 'internal' variables were defined as those directly involved with sample preparation, such as CB components or NBH properties. The extent to which the effects of the external and internal variables were investigated was dependent on whether results obtained were in support of what had been previously shown in the literature.

3.2.3.1 External variables

External variables that were investigated included the following: 1) duration of PMCA, 2) incubation of the samples at 37^{o}C prior to PMCA, 3) the requirement of sonication for amplification, 4) sonication intensity, 5) temperature (*i.e.*, investigating the use of traditional PMCA versus the use of a water bath with continuous recirculation of water in the PMCA reservoir), and 6) microtubes used for PMCA. These variables were chosen due to discrepancies in the literature between the laboratories utilizing PMCA (Table 3.2 and Table 3.3) and through consultation with other researchers working on optimizing and adapting PMCA in their respective laboratories.

3.2.3.1.1 *Duration of PMCA*

This phase of optimization assessed the impact associated with varying the duration of PMCA on the overall level of *in vitro* amplification. Both, Saborio *et al.* (204) and Saa *et al.* (203) had shown that amplification rate was proportionate to the number of incubation/sonications cycles performed. The effect of duration on PrP^{Sc} amplification *in vitro* from independent replicates (on the same sonicator) are provided in Figure 3.3 and summarized in Table 3.4. The optimal length of time for the PMCA assay was determined to be 16 hours (Figure 3.3c, Table 3.4), whereby amplification approximated 2 $\log_{10}$ in one round of PMCA. Both shortened (3 and 6 hours, Figure 3.3a and 3.3b, respectively) and extended periods of PMCA (24, 48 and 68-72 hours, Figure 3.3d, 3.3e and 3.3f, respectively) resulted in consistently lower levels of amplification than that observed for 16 hours (32 cycles). Thus the duration of the basic protocol was modified to 16 hours (*i.e.*, overnight) based on higher average amplification achieved, as well as providing more consistency and reproducibility in results. From a laboratory standpoint the overnight duration was convenient and logical; PMCA samples could be set-up and placed on the PMCA machine by the end of day one and retrieved early the following morning and processed.

Table 3.4 Summary of estimated amplification based on duration of PMCA.

	Duration of PMCA					
	3 hr	**6 hr**	**16 hr**	**24 hr**	**48 hr**	**68/72 hr**
Replicate (i)	~5x	5x	25x	5x	25x	5x
Replicate (ii)	5x	25x	125x	5x	25x	25x
Replicate (iii)	~5x	5x	125x	5x	5x	25x
Average amplification	~$1x10^1$x	~$1x10^1$x	~$9x10^1$x	~$1x10^1$x	~$2x10^1$x	~$2x10^1$x

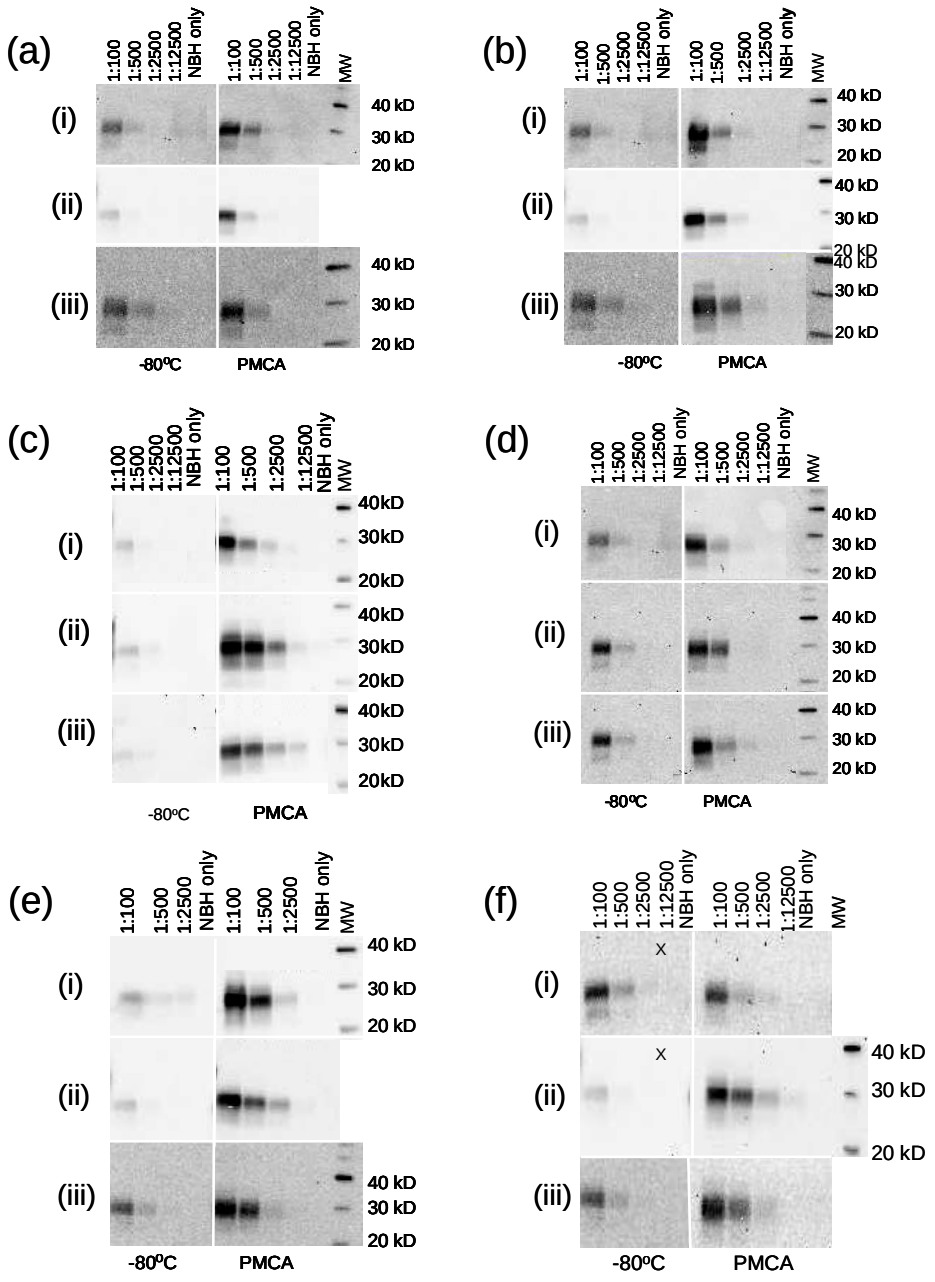

Figure 3.3 Western blots demonstrating the effect of duration of PMCA on PrP^{Sc} amplification. Durations tested included; (a) 3 hours, (b) 6 hours, (c) 16 hours, (d) 24 hours, (e) 48 hours, and (f) 68-72 hours, each with three replicates (i, ii, iii) from independent experiments. Infectious brain homogenate (IBH) was serially diluted (1:100-1:12500) into normal brain homogentate (NBH) and treated with Proteinase K (PK). Lanes labeled 'X' represent no sample. Lanes labeled 'MW' represents the molecular weight markers at 40, 30, 20 kD.

3.2.3.1.2 *Pre-incubation or period of acclimation of PMCA samples in sonicator*

A pre-incubation or acclimation step was generally part of the PMCA protocol for most laboratories. Manual PMCA methods specified a 1 hour pre-incubation period (37,41,201), which was shortened to 30 min with the introduction of the automated sonicator (41). This pre-incubation step consisted of placing the samples for PMCA in the water reservoir of the sonication horn for 30 min prior to the initial burst of sonication, however a technical recommendation in Castilla *et al.* (37) suggested that if PrP^{Sc} is highly concentrated and/or aggregated (such as low dilutions of experimental scrapie brain homogenate) it is advisable to begin with sonication instead of incubation. In this thesis work, pre-incubation of samples was not deemed necessary based similar or slightly better amplification levels without the pre-incubation period (Figure 3.4). For this reason the pre-incubation step was eliminated for the optimized PMCA protocol.

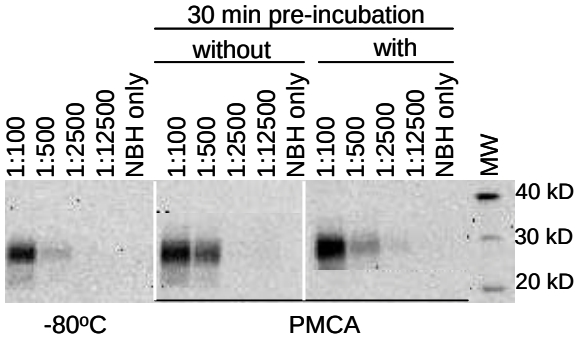

Figure 3.4 Western blot comparison demonstrating the effect of pre-incubation of samples prior to PMCA. Pre-incubation refers to 30 min at ~37°C prior to the first burst of sonication with PMCA. Infectious brain homogenate (IBH) was serially diluted (1:100-1:12500) into normal brain homogentate (NBH) and treated with Proteinase K (PK). Lane labeled 'MW' represents the molecular weight markers at 40, 30, 20 kD.

3.2.3.1.3 *Requirement of sonication*

Certain *in vitro* conversion methods adapted from the PMCA assay, such as QuIC (7), rely on vigorous mixing of samples to yield amplification of PrP^{Sc} *in vitro*. Lucassen *et al.* (143) had also determined that minimal amplification (10x) could be achieved with incubation only at 25°C or 37°C. In order to determine whether sonication was a requirement for PMCA, parallel samples were incubated at ~37°C only and compared to amplification achieved with samples exposed to an equivalent amount of time (*i.e.*, 16 hours) using PMCA. Samples placed in a 37°C water bath for 16 hours resulted in less amplification than samples processed on the PMCA machine (Figure 3.5). Incubation of samples at 37°C also resulted in little or no amplification compared to controls (-80°C). Any amplification that was observed was substantially less compared to samples exposed to PMCA for the same amount of time (Figure 3.5). On average, >25x amplification was achieved with the use of PMCA (*i.e.*, sonication as well as incubation) compared to incubation alone, deeming sonication necessary in the optimized PMCA protocol.

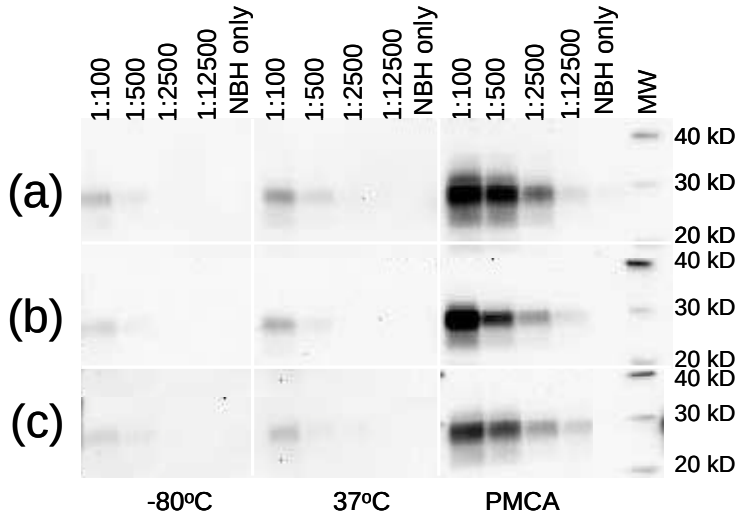

Figure 3.5 Western blots demonstrating the necessity of sonication for PrP^{Sc} amplification. Three replicates (a), (b), and (c) were from independent experiments. Incubation only (37°C) samples were kept at 37°C for the same amount of time as PMCA samples that were subjected to cycles of sonication while incubating at 37°C. Lane labeled 'MW' represents the molecular weight markers at 40, 30, 20 kD.

3.2.3.1.4 *Sonication intensity*

Sonication was a requisite for amplification of PrP^{Sc} by the PMCA assay, therefore sonication intensity was the next parameter addressed in optimization. A potency of 80-100% for aPMCA systems had been previously reported to be optimal for 263K (6,41,201). Castilla *et al.* (41) suggested that an increase in sonication intensity may be required if the number of samples exceeded 60% of the sample rack capacity and that after extended use the sonicator intensity may diminish over time (58). To determine the impact of an increase or decrease in sonication intensity, power settings of 40, 60 and 100% were compared to 80% used in the basic protocol (Figure 3.6).

A power setting of 6 (*i.e.*, 60%) was determined optimal for PMCA, consistently resulting in an equivalent or higher level of amplification compared to all other power settings across independent experiments (Table 3.5). Although power settings equivalent to 80% and 100% sonication resulted in reasonable levels of amplification (~$8x10^1$x and $9x10^1$x amplification compared to controls, respectively), sonication at this high power induced splashing of the sample into the lid of the microtubes, and in some cases the lids on the microtubes popped open. Sample contents left in the lid during repetitive cycles of PMCA may not receive adequate sonication throughout the single round of PMCA, and coupled with risks associated with lids popping open, a lower power setting was preferred. Power settings of 40% yielded lower amplification rates compared to 60% (Figure 3.6 and Table 3.5). Although, variability between experiments was still apparent, a power setting of 6 (*i.e.*, 60%) seemed to result in fewer anomalies or inconsistencies in amplification and was therefore chosen as the optimal sonication intensity.

Table 3.5 Summary of estimated amplification of PMCA with varying intensity of sonication power (40, 60, 80, and 100%).

	Sonication intensity			
	40%	**60%**	**80%**	**100%**
Replicate (i)	-	>125x	>25x	>125x
Replicate (ii)	-	625x	>125x	125x
Replicate (iii)	-	25x	5x	>25x
Replicate (iv)	5x	125x	-	-
Replicate (v)	25x	125x	-	-
Replicate (vi)	>25x	25x	-	-
Replicate (vii)	125x	25x	-	-
Replicate (viii)	25x	125x	-	-
Average amplification	~$4x10^1$x	~$2x10^2$x	~$8x10^1$x	~$9x10^1$x

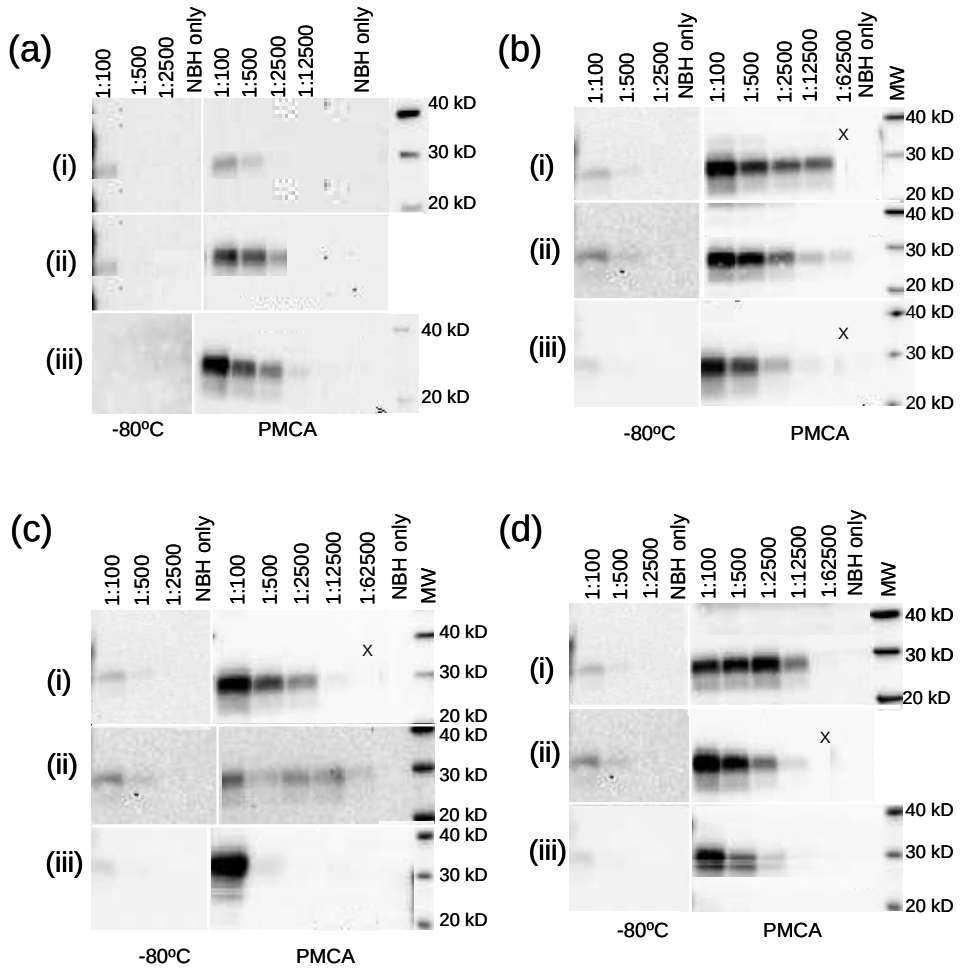

Figure 3.6 Western blots demonstrating the effect of PMCA sonication intensity on PrP^{Sc} amplification. Four sonication intensities were tested; (a) 40%, (b) 60%, (c) 80% and (d) 100%, each with three replicates (i), (ii) and (iii) from independent experiments. Infectious brain homogenate (IBH) was serially diluted (1:100-1:62500) into normal brain homogentate (NBH) and treated with Proteinase K (PK). Lanes labeled 'X' represent no sample. Lanes labeled 'MW' represents the molecular weight markers at 40, 30, 20 kD.

3.2.3.1.5 *Temperature*

Evidence that shorter durations (16 hours) of PMCA enhanced amplification suggested that the extended periods of incubation may compromise

sample integrity of the NBH/sample preparation and possibly that temperature may have an effect on the stability of the amplification process. The basic PMCA protocol employed a temperature of 37^{o}C, however a previous report emphasized that prolonged incubation at 37^{o}C could result in deleterious effects (37). Preliminary assessment of temperature fluctuations before and after a single burst of sonication displayed a slight increase in temperature with each burst (~2-3 ^{o}C) and when extended to overnight repetitive cycles of sonication the water reservoir temperature could reach as high as 42-43^{o}C (Table 3.6). Atarashi *et al.* (6) demonstrated efficient conversion of recombinant PrP^{C} with the use of a circulating water bath to accurately maintain the water reservoir temperature during the PMCA assay. Therefore, a peristaltic pump system was devised to deliver and circulate water between a water bath and the PMCA reservoir while maintaining the desired temperature +/- 1-2 ^{o}C. In a trial overnight PMCA run, the peristaltic pump with water bath system was successfully able to maintain a temperature of ~37^{o}C, after 2 days (40 hours) of continuous running the temperature drifted to 40^{o}C (Table 3.6).

The two temperature control systems (*i.e.*, peristaltic pump and standard incubator) were tested for amplification efficiency by PMCA. More consistent and increased amplification was achieved with the standard incubator system in all three replicates of the experiment (Figure 3.7). Increased temperature fluctuations in the standard incubator system did not seem to have a detrimental effect on the amplification rates of PrP^{Sc} by PMCA.

Table 3.6 Temperature differences between PMCA water reservoir temperature with incubator or peristaltic pump with water bath system.

	Water reservoir temperature (°C)		
	Time zero	**After 16 hours**	**After 40 hours**
Incubator	32.5	43	44
Peristaltic pump with water bath	35.5	37	40

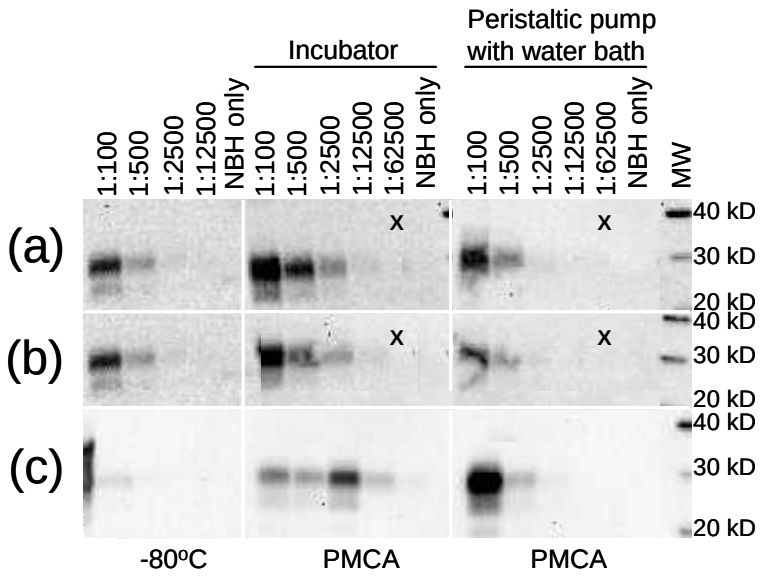

Figure 3.7 Western blot comparison demonstrating PrP^{Sc} amplified using a standard incubator (incubator) or peristaltic pump (peristaltic pump with water bath) to control for temperature during PMCA. Replicates (a), (b) and (c) were from independent experiments. Infectious brain homogenate (IBH) was serially diluted (1:100-1:62500) into normal brain homogentate (NBH) and treated with Proteinase K (PK). Lanes labeled 'X' represent no sample. Lane labeled 'MW' represents the molecular weight markers at 40, 30, 20 kD.

3.2.3.1.6 *Sample vessels*

The sample vessel type used for PMCA was also tested for efficiency of amplification. Atarashi *et al.* (6) specifically mentioned the importance of using certain microtubes for PMCA, whereas other published methods did not specify,

but emphasized the use of thin walled tubes to obtain the most effective penetration of ultrasonic waves (37,41,201). In personal communication with other laboratories involved in development of PMCA, the use of attached-cap, thin walled 0.2 ml microtubes was emphasized (Nalgene/Nunccatalogue #250875, Nalgene/Nunc International, Rochester, NY, USA) over strip-cap formats.

An experiment was set-up to determine the variation in PrP^{Sc} amplification associated with attached-cap (Nalgene/Nunc catalogue #250875) versus strip-cap (Nalgene/Nunc catalogue #248161) microtubes using PMCA. Little or no variation in amplification efficiency was observed between these two types of sample vessels (Figure 3.8). Therefore the use of the attached-cap microtubes was maintained for the optimized PMCA protocol.

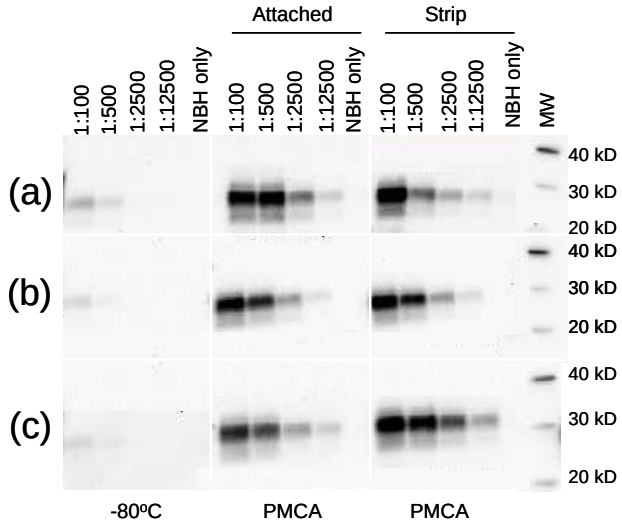

Figure 3.8 Western blot comparison of PrP^{Sc} amplification using attached-cap and strip-cap microtube sample vessels. Replicates (a), (b) and (c) were from independent experiments. Infectious brain homogenate (IBH) was serially diluted (1:100-1:12500) into normal brain homogentate (NBH) and treated with Proteinase K (PK). Lane labeled 'MW' represents the molecular weight markers at 40, 30, 20 kD.

3.2.3.2 Internal variables

Internal variables, referring to variables having a direct effect on the sample itself, were modified to determine their impact on the PMCA assay. These variables included: 1) the requirement of daily preparation of the CB, 2) concentration of detergent (Triton X-100), 3) use of perfused compared to non-perfused hamster brains in CB preparation, 4) the necessity of freshly prepared versus previously frozen NBH, and 5) the effect of freeze-thawing of the IBH prior to dilution series set-up. These variables were chosen based primarily on recommendations in technical procedures presented by the inventors of PMCA (37,41,201).

3.2.3.2.1 *Requirement of daily preparation of conversion buffer*

Based on previously published data, the chemical composition of the CB used in PMCA can dramatically affect the efficiency of the PrP^{Sc} amplification (41). For this reason, no direct changes were made in the buffer composition of PMCA for the optimized assay, but the stability of CB preparations over time was investigated. To determine whether storage of the reagents would have an effect on the *in vitro* amplification of PrP^{Sc} by PMCA, CB was prepared daily (fresh CB) and compared to CB preparations that were $\geq$1 week old (old CB). In only one of three independent trials, preparation of fresh CB led to a slightly greater amplification of PrP^{Sc} than the stored preparation (old CB). Figure 3.9 shows the comparison of 3 trials of CB prepared just prior to experimental set-up (*i.e.*, 'fresh') with CB prepared $\geq$1 week prior to the experiment (one week being an appropriate time frame to complete any experiments that required several days of

PMCA experiments/rounds). These findings confirmed that daily preparation of the CB was not imperative. For standardization and convenience, CB was typically prepared in a large enough volume to allow for set-up of each individual experiment in its entirety or for multiple experiments if necessary.

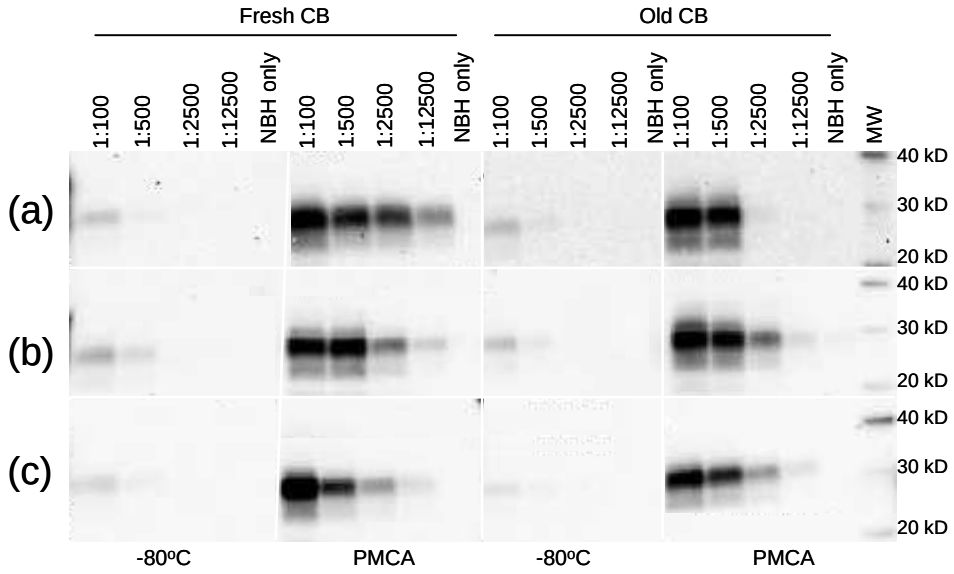

Figure 3.9 Western blot comparison of PrP^{Sc} amplification based on conversion buffer (CB) prepared the day of use (fresh CB) or previously (old CB). Replicates (a), (b) and (c) were from independent experiments. Infectious brain homogenate (IBH) was serially diluted (1:100-1:12500) into normal brain homogentate (NBH) and treated with Proteinase K (PK). Lane labeled 'MW' represents the molecular weight markers at 40, 30, 20 kD.

3.2.3.2.2 *Detergent concentration*

Determining the appropriate detergent concentration for PMCA was necessary for several reasons. Firstly, Triton X-100 permeates cell membranes, allowing for solubilization of important co-factors and enzymes required for the conversion process. Secondly, the wetting properties of the detergent were

exploited to ensure that after the sonication burst the aerosolized droplets would 'slide' back into the bottom of the microtubes so that the newly formed aggregate seeds would again be in contact with excess PrP^{C} for further conversion. Thirdly, too much detergent can lead to excessive bubble formation, believed to impair transmission of sonication waves (41). Fourthly, Triton X-100 concentrations in excess of 1% may inactivate PK hydrolysis during sample processing after PMCA (35). Personal communications with other research groups revealed unique approaches to dealing with optimization of detergent concentrations. For example, in one laboratory, batches of CB were prepared with 0.9%, 1.0% and 1.1% Triton X-100 and tested for compatibility against a single NBH preparation for amplification of PrP^{Sc} by PMCA. The CB preparation yielding the highest level of amplification was used and all others discarded.

The effect of Triton X-100 concentrations on PrP^{Sc} amplification by PMCA from independent trials is provided in Figure 3.10. A final concentration of 1% Triton X-100 in the CB was maintained (in agreement with the literature) based on the minimal variation in amplification observed with the concentrations tested.

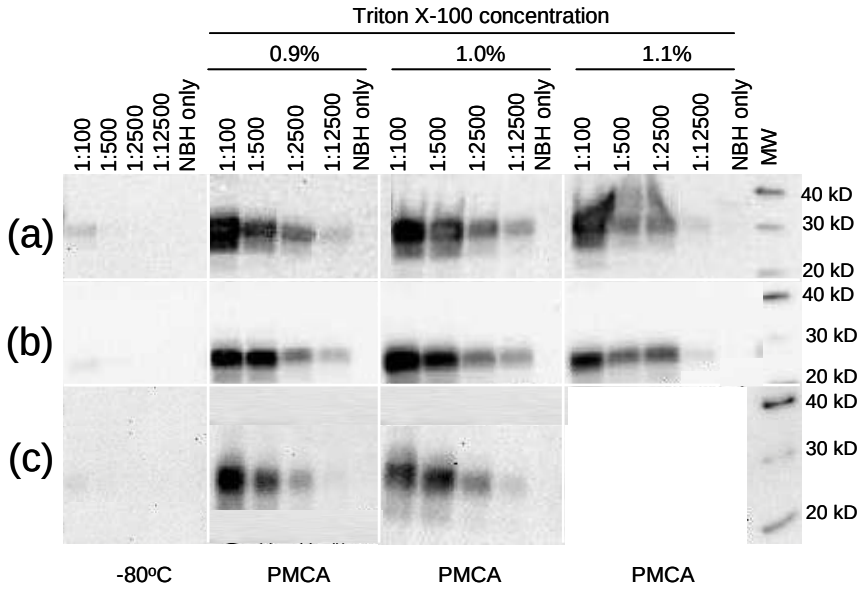

Figure 3.10 Western blot comparison of PrP^{Sc} amplification based on variation in Triton X-100 concentrations (0.9, 1.0 and 1.1%) in the conversion buffer (CB) preparations. Replicates (a, b and c) were from independent experiments. Infectious brain homogenate (IBH) was serially diluted (1:100-1:12500) into normal brain homogentate (NBH) and treated with Proteinase K (PK). Lane labeled 'MW' represents the molecular weight markers at 40, 30, 20 kD. A different gel format (NuPAGE) was used for replicate (c).

3.2.3.2.3 *Freezing of samples prior to PMCA*

Freeze-thawing of samples has the potential to promote disruption of cell membranes, co-factors and/or enzymes and Saa *et al.* (201) noted that frequent freezing-thawing of NBH significantly reduced amplification. To investigate this, two identical PMCA dilution series were set-up with one set frozen at -80^{o}C and allowed to thaw prior to application of the PMCA conditions, and the other set kept at 4^{o}C or on ice until PMCA (*i.e.*, refrigerated). Trends for the two replicates were similar (Figure 3.11) and no observable effect of freezing samples prior to PMCA could be seen. Therefore the laborious nature and time constraints

associated with the daily preparation of an NBH substrate followed by set-up of the experimental dilution series were not justified. Furthermore, freezing samples prior to further manipulation or processing eliminated the impact of laboratory scheduling or infrastructure constraints (*i.e.,* lack of equipment, work load of researcher, laboratory shutdowns).

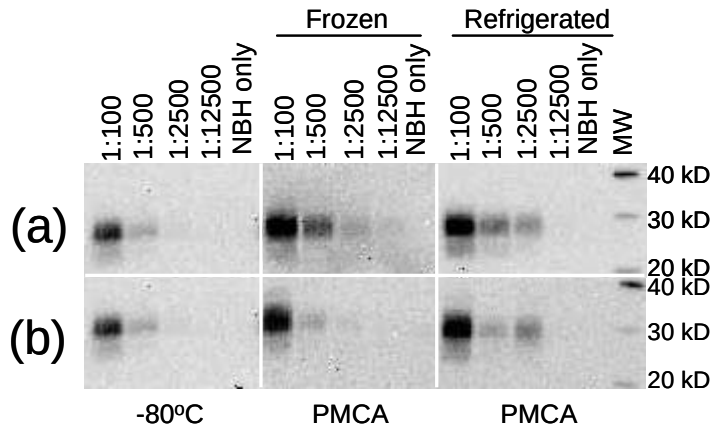

Figure 3.11 Western blot comparison of the amplification of PrP^{Sc} based on freezing or refrigerating experimental dilution series prior to PMCA. The term 'frozen' refers to samples frozen once at -80^{o}C prior to PMCA, while 'refrigerated' samples were kept at 4^{o}C until PMCA was performed. Replicates (a) and (b) were from two independent experiments. Infectious brain homogenate (IBH) was serially diluted (1:100-1:12500) into normal brain homogentate (NBH) and treated with Proteinase K (PK). Lane labeled 'MW' represents the molecular weight markers at 40, 30, 20 kD.

3.2.3.2.4 *Perfused vs non-perfused normal hamster brains*

A small comparison of PMCA using perfused and non-perfused brains in the NBH preparation was conducted in an effort to determine the importance of the clearance of blood and its' components from hamster brains prior to use.

Castilla *et al.* (41) stressed the importance of removal of blood components as they seriously affected the amplification process. Thus, virtually all studies in which PMCA is used the researchers specify the use of perfused brains for preparation of NBH. Interestingly the use of non-perfused hamster brains showed little difference compared to perfused brains in this small trial (Figure 3.12). This suggested that the inhibitory effects reported by blood or components of blood may not have as detrimental effect on the PMCA amplification as would be expected. However, in an effort to maintain consistency with the PMCA literature methods and to circumvent the potential of future problems with non-perfused brains with application of the PMCA protocol to plasma, perfused brains were utilized in the optimized PMCA assay.

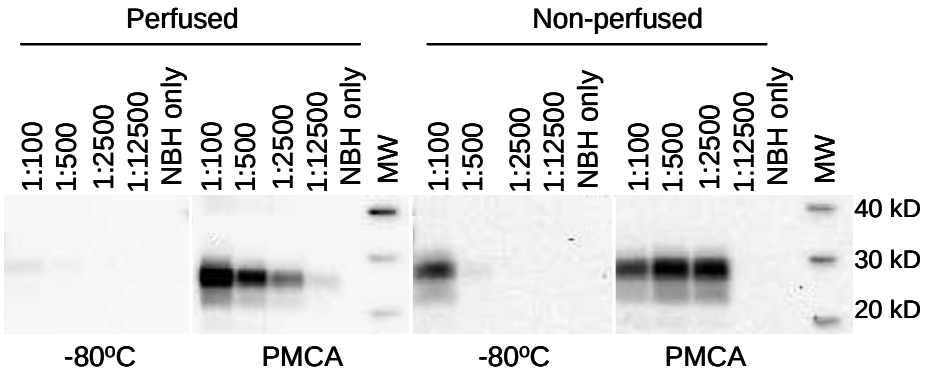

Figure 3.12 Western blot demonstrating the effect of perfused and non-perfused normal brains on the amplification of PrP^{Sc} by PMCA. Normal brains were either perfused with PBS and 5mM EDTA (perfused) or not (non-perfused) at the time of sacrifice. Infectious brain homogenate (IBH) was serially diluted (1:100-1:12500) into normal brain homogentate (NBH) and treated with Proteinase K (PK). Lanes labeled 'MW' represents the molecular weight markers at 40, 30, 20 kD.

3.2.3.2.5 *Freeze-thaw of IBH*

Lastly, the effect of freeze-thaw cycles of the IBH on amplification of PrP^{Sc} was considered. The stability of PrP^{Sc} to mediate template-directed amplification after up to 20 freeze-thaw cycles had been reported (41), but multiple freeze-thaws affected both the size and number of the large PrP^{Sc} aggregates, leading to amplification variability due to sample dilution errors. Figure 3.13 displays the results of freeze-thaw cycles and their effect on PrP^{Sc} amplification. A slight decline in amplification rate was observed after 25 freeze-thaw cycles which was in agreement with reports in the literature (41).

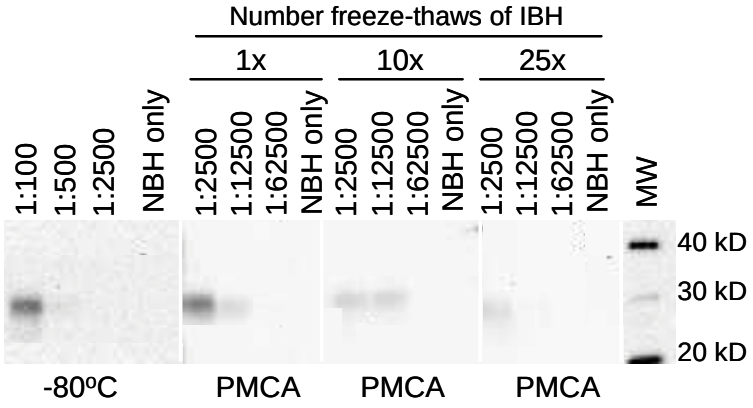

Figure 3.13 Western blot comparison effect on PrP^{Sc} amplification with freeze-thaw cycles of the infectious brain homogenate (IBH) (1x, 10x, and 25x) prior to PMCA experimental dilution set-up. Infectious brain homogenate (IBH) was serially diluted (1:100-1:62500) into normal brain homogentate (NBH) and treated with Proteinase K (PK). Note that the dilution series starts at 1:2500 for the PMCA samples. Lane labeled 'MW' represents the molecular weight markers at 40, 30, 20 kD.

3.2.3.3 Serial PMCA (sPMCA)

Numerous laboratories had reported increased amplification with serial dilution of the PMCA product (sPMCA) into fresh substrate (NBH with co-factors) (21,41,42,134,182,202,203,220,251). However variations existed in transfer volumes (or dilution) to subsequent rounds and the ability of successive rounds to significantly increase amplification (6,21,42,134,182,203). Serial PMCA was investigated to determine the degree of impact on PrP^{Sc} amplification in the context of the basic PMCA assay.

The effect of dilution of the first round of PMCA (1^{o} PMCA) product into fresh NBH and subjecting to an additional round of PMCA (2^{o} PMCA) is shown in Figure 3.14. A 1:10 dilution was chosen based on previous published literature with 263K hamster brain homogenates (41,42). Although previously longer durations resulted in less PrP^{Sc} amplification with longer time frames (Table 3.4), the 2^{o} PMCA with no dilution (*i.e.*, longer duration only) resulted in approximately 1 $\log_{10}$ greater amplification over the 1^{o} PMCA round. A 1:10 dilution of the 1^{o}PMCA product into fresh NBH however, resulted in an even larger increase in amplification, equivalent to >3 $\log_{10}$. The level of amplification detected after 2^{o} PMCA was calculated by dividing the highest detectable titre (band of similar intensity to the control) of the 2^{o} PMCA by the titre of the -80^{o}C control displaying PrP^{Sc} signal (1:100), and multiplying by the dilution between primary and secondary rounds of PMCA (1:10), *i.e.*, 12500/100 * 10 = 1250x.

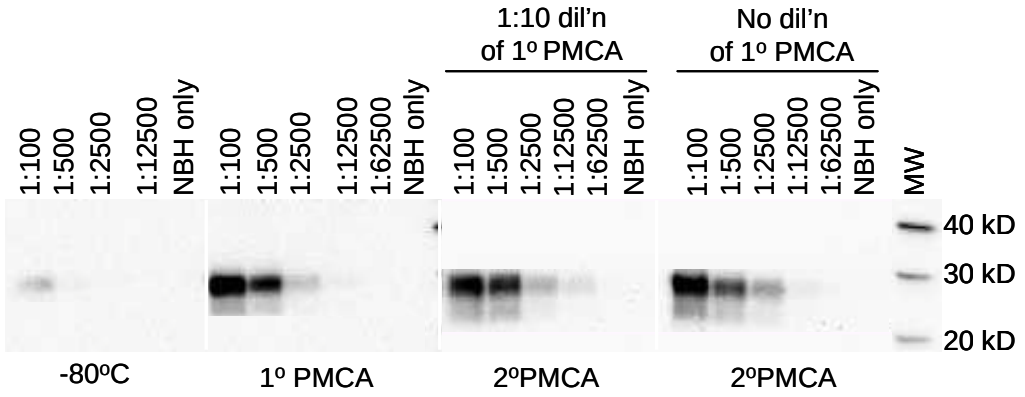

Figure 3.14 Western blot demonstrating the effect of PrP^{Sc} amplification after two rounds of sPMCA with and without dilution of the 1^{o} PMCA product. The primary round PMCA product was either subjected to 2^{o} PMCA without dilution into fresh NBH (no dil'n) or diluted into fresh NBH (1:10 dil'n). Infectious brain homogenate (IBH) was serially diluted (1:100-1:62500) into normal brain homogentate (NBH) and treated with Proteinase K (PK). Lane labeled 'MW' represents the molecular weight markers at 40, 30, 20 kD.

The dilution of the primary product used in subsequent rounds of sPMCA is dependent on the purpose of the study and the original PrP^{Sc} dilution (41). Lower dilutions (1:5) were generally utilized when PrP^{Sc} concentrations were expected to be limiting and higher dilutions (1:10-1:1000) when the initial amount of PrP^{Sc} was relatively high (brain) (21,42,134,202,213). The results of numerous replicates of 1^{o} PMCA, along with subsequent 2^{o} PMCA at varying dilution were calculated and tabulated in Table 3.7, with one representative WB replicate depicted in Figure 3.15. Dilution of the 1^{o} PMCA product with all dilutions investigated produced equivalent or more amplification than with a single round of PMCA, reinforcing the advantage of diluting primary products into fresh NBH and performing a 2^{o} PMCA. Based on the calculated average amplification, as

well as consistency amongst replicates, a 1:10 or 1:100 dilution of primary product into secondary reactions was deemed optimal (although variability amongst the individual replicates was apparent). Although high levels of amplification were also seen with higher dilutions (1:100, 1:1000), previously published reports emphasized the need for a 'minimum seed requirement' for PMCA (146), thus dilutions lower than 1:10 were subsequently not used in the remainder of this thesis.

Table 3.7 Overall amplification after 2^o PMCA for various dilutions (1:5, 1:10, 1:100 and 1:1000) of the 1^o PMCA product.

	Total overall amplification[a]				
	1^o PMCA	**2^o PMCA**			
		1:5	**1:10**	**1:100**	**1:1000**
Replicate (a)	125x	625x	1250x	>2500x	5000x
Replicate (b)	125x	625x	1250x	2500x	<1000x
Replicate (c)	25x	125x	50x	2500x	<1000x
Replicate (d)	25x	3125x	250x	2500x	5000x
Replicate (e)	125x	625x	1250x	2500x	5000x
Replicate (f)	625x	3125x	31250x	5000x	1000x
Average amplification	~$2x10^2$x	~$1x10^3$x	~$6x10^3$x	~$3x10^3$x	~$3x10^3$x

[a] total overall amplification refers to the amount of amplification after the number of PMCA rounds ran (1^o or 2^o) taking into account dilution factor

Up to 3 rounds of PMCA were performed during the optimization of sPMCA. The amplification rates from performing numerous independent replicates of successive rounds of PMCA are shown in Table 3.8. The table numerically displays the variability of individual replicates associated with PMCA. Investigating the increased amplification over the previous round led to an average of ~$3x10^1$x (between 10-50x) greater amplification after the 2^o PMCA, with similar increases after 3^o PMCA.

Table 3.8 Average amplification achieved with additional rounds of PMCA.

	Round of PMCA[a]		
	1°	**2°**	**3°**
Replicate (a)	125x	1250x	12500x
Replicate (b)	125x	1250x	62500x
Replicate (c)	25x	250x	12500x
Replicate (d)	5x	250x	12500x
Replicate (e)	625x	31250x	12500x
Average amplification	~$2x10^2$x	~$7x10^3$x	~$2x10^4$x
Average increase in amplification from previous round		~$3x10^1$x	~$3x10^1$x

[a] the previous round PMCA product was diluted 1:10 into fresh NBH prior to the 2° and 3° PMCA

3.2.3.4 Variability within and between PMCA machines

Variability of position within the PMCA machine had been evaluated by other laboratories and found to have little effect on the amplification rate (203,210). Figure 3.16 displays the results of variability within a single PMCA machine over one experiment, confirming apparent, but minimal variation dependent on location within the microplate horn. This underscored the importance of multiple replicates (minimum two, preferably three) to accurately assess amplification results associated with PMCA.

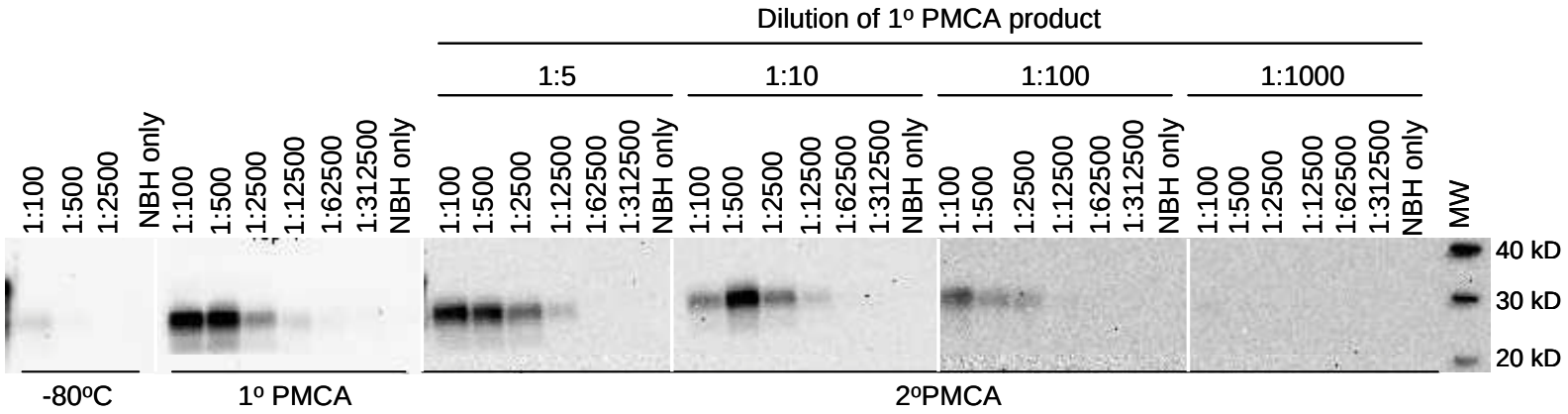

Figure 3.15 Representative Western blot demonstrating the effect on PrP^{Sc} amplification with dilution of the 1^{o} PMCA product (1:5, 1:10, 1:100 and 1:1000) for 2^{o} PMCA. 1^{o} PMCA product was diluted into fresh normal brain homogenate (NBH). Infectious brain homogenate (IBH) was serially diluted (1:100-1:312500) into normal brain homogentate (NBH) and treated with Proteinase K (PK). Lane labeled 'MW' represents the molecular weight markers at 40, 30, 20 kD. This figure corresponds to replicate (b) in Table 3.7.

Since the efficiency of PMCA machines was known to decline with extended use, variability between machines was likely to occur (41). To determine the extent of variability between different machines several independent replicates were conducted between two machines (Figure 3.17). Reproducible amplification was achieved with the use of both machines and within each replicate minimal variation in amplification (~5x) between machines was observed. The degree of variation if a higher level of amplification was achieved could not be assessed. Caution was used when comparing and analyzing amplification from multiple PMCA machines.

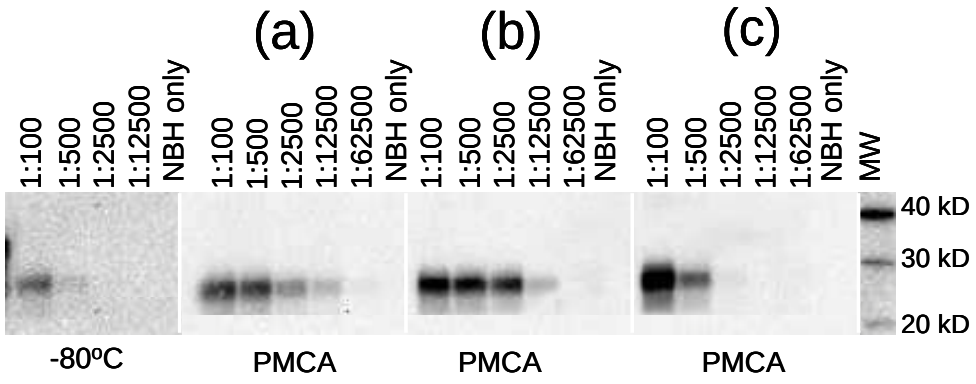

Figure 3.16 Western blot comparison of variability within a single PMCA machine. Three replicates, (a), (b) and (c) were from independent experiments. Infectious brain homogenate (IBH) was serially diluted (1:100-1:62500) into normal brain homogentate (NBH) and treated with Proteinase K (PK). Lane labeled 'MW' represents the molecular weight markers at 40, 30, 20 kD.

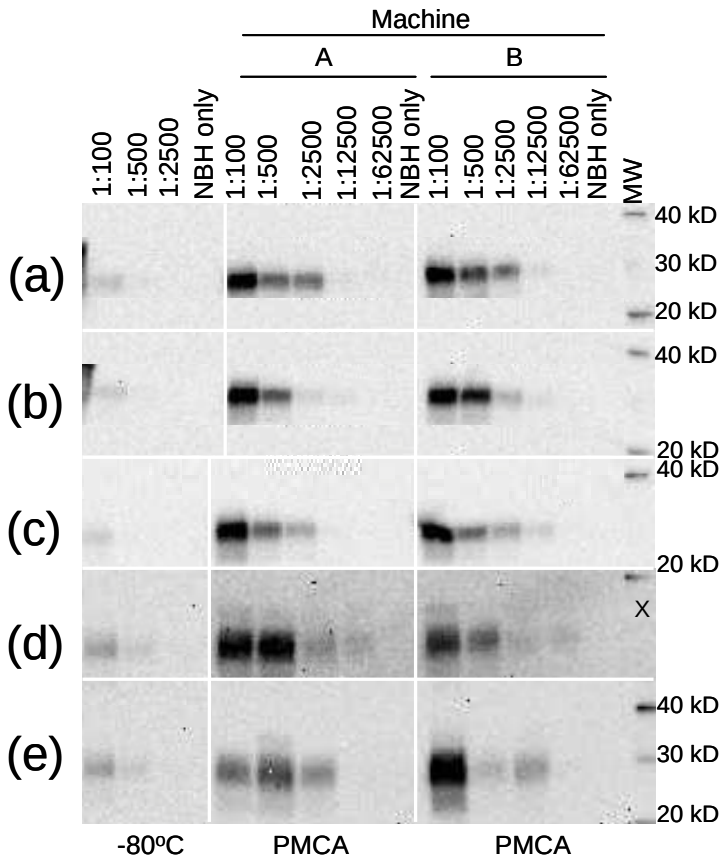

Figure 3.17 Western blot comparison of variability between two PMCA machines (labeled Machine A and B). Replicates (a) – (e) were from independent experiments. Infectious brain homogenate (IBH) was serially diluted (1:100-1:62500) into normal brain homogentate (NBH) and treated with Proteinase K (PK). Lane labeled 'MW' represents the molecular weight markers at 40, 30, 20 kD.

3.2.4 *Standard and optimized PMCA assay*

The specific parameters of the optimized PMCA were determined through evaluation of the effect on amplification from the numerous external and internal variables described, including conditions of sPMCA. Table 3.9 highlights the variations made to the basic protocol during the optimization process described in this thesis. The optimized PMCA protocol, was capable of reaching ~3 $\log_{10}$

amplification after 2^o PMCA (64 cycles, 32 hours), displaying similar levels of amplification as: a) Castilla *et al.* (42) after 1^o PMCA of 140 cycles (70 hours) in the buffy coat fraction of blood, b) Atarashi *et al.* (6) after 1^o rPMCA of 24 cycles (24 hours) in scrapie brain homogenates, and c) Jones *et al.* (120) after 1^o PMCA of 48 cycles (24 hours) in vCJD brain (Table 3.1). The optimized PMCA protocol was subsequently used to address the second objective of this thesis, application of PMCA to detect prions in blood plasma (Chapter 4).

Table 3.9 Overview of the basic PMCA protocol to the optimized PMCA protocol.

	BASIC PMCA PROTOCOL	**OPTIMIZED PMCA PROTOCOL**
Duration/cycles	24 hours/48 cycles	16 hours/32 cycles
Sonication intensity	80%	60%
Temperature/incubation	Sonicator/bench top incubator	Sonicator/bench top incubator
Sample vessel (strip- or attached- cap)	Nalgene/Nunc 248161 Attached-cap	Nalgene/Nunc 248161 Attached-cap
CB prepared daily	Always	Not necessary
Detergent (Triton X-100) concentration	1%	1%
NBH froze prior to PMCA	No	Always
Perfused or non-perfused normal brains	Perfused	Perfused
Freeze-thaw IBH	<20x	<20x
Additional round PMCA	sPMCA	sPMCA (2 rounds)
Dilution in 2^o PMCA	1:10	1:10
Estimated amplification	~1 $\log_{10}$ (1 round)	~3 $\log_{10}$ (2 rounds)

4 ADAPTATION OF OPTIMIZED PROTEIN MISFOLDING CYCLIC AMPLIFICATION PROTOCOL TO DETECTION OF PRIONS IN HAMSTER PLASMA

4.1 Introduction

In 1998, the infectious nature of blood associated with TSEs was clearly demonstrated via animal bioassay (26). Subsequently, fractionation of blood into individual constituents displayed that infectivity was associated with the buffy coat, plasma and Cohn fractions of blood (24,232). Unfortunately post-mortem diagnostics for prion detection have been unsuccessful when applied to blood due to the significantly lower levels of PrP^{Sc} circulating in blood (100-1000x lower) than are found in the brain (93).

The introduction of *in vitro* prion amplification techniques, such as PMCA, when coupled with conventional diagnostic assays (*i.e.,* Western blot, ELISA) have the potential to significantly improve detection sensitivities associated with extremely low levels of infectious prions that may be circulating in body fluids such as plasma. Application of the PMCA assay to various matrices, including blood and urine, and detection of PrP^{Sc} pre-symptomatically exemplifies the potential of this ultrasensitive tool for diagnostic purposes (6,42,90,220). In 2005, Castilla *et al*. (42), using the PMCA assay, were the first laboratory to demonstrate successful detection of PrP^{Sc} in the buffy coat fraction of blood. Subsequently over the last 3 years, other laboratories have applied the PMCA assay, or slight modifications thereof, to plasma (159,230), blood leukocytes (237), platelets (121) and very recently whole blood (199).

When this thesis was initialized (2006), the only successful application of the PMCA assay to blood was associated with the 263K hamster buffy coat separated from whole blood at end stages of disease (42). Technical publications by Castilla *et al*. (37,41) and Saa *et al*. (201) had stressed the importance of removal of blood from brains during extraction that were destined to be used as PMCA substrate, observing that small amounts of plasma or serum inhibit the PMCA reaction, along with the PK digestion used during detection. When Murayama *et al*. (159) applied the PMCA assay, using a different automated sonicator (ELESTEIN 070-GOT, Elekon Science Corp., Chiba, Japan) to Sc237 hamsters, they were able to replicate Castilla *et al*. (42) results in buffy coat, but were only successful in the detection of PrP^{Sc} in a portion of the plasma samples tested. After 160 cycles of PMCA, two of the four plasma samples collected 60 dpi displayed a PrP^{Sc} resistant band, but an additional 120 PMCA cycles (280 cycles) did not lead to the PrP^{Sc} detection in the two remaining plasma samples. They concluded that PrP^{Sc} was present in blood during both the symptomatic and terminal stages of disease, however the lower levels of PrP^{Sc} associated with plasma could not be detected as readily as in buffy coat (PrP^{Sc} was detected in all four buffy coat samples from 60 dpi after 160 cycles of PMCA). This important publication provided evidence that the PMCA platform was modifiable to the plasma component of blood.

The fractions of blood which have higher levels of infectivity associated with them (*i.e.*, buffy coat and leukocytes) require pelleting and/or freeze-thaw cycles prior to PMCA application. Studies with urine had demonstrated that pre-

concentration of PrP^{Sc} prior to PMCA was detrimental to PrP^{Sc} recovery (24,42,90,159,237). Similar to urine preparations, blood preparation protocols have a significant effect on the normal PrP^{C} concentrations, and therefore are likely to have an effect on recovery of PrP^{Sc} in plasma (94,262). Thus far only 2 studies report detection of PrP^{Sc} in plasma. The first study, utilized low dilutions of plasma (*i.e.*, 1:10 and 1:100) distinguishing only 2 of 4 infected animals after 280 cycles of PMCA (159). The second, used limited-sPMCA (only 40 cycles) followed by immunoprecipitation with a monoclonal PrP antibody (8E9) and detection with surround optical fiber immunoassay (SOFIA) capable of detection of PrP^{Sc} in protease-untreated samples (199). Conventional diagnostics (*i.e.*, capture ELISA and WB) were not sensitive enough to detect the PrP^{Sc} associated with plasma after limited-sPMCA or the combination of limited-sPMCA followed by immunoprecipitation in this study (199).

Using the optimized PMCA conditions outlined in Chapter 3 of this thesis, adaptation and application of PMCA to plasma was initiated. Initially the effectiveness of the WB for detection of PrP^{Sc} in the presence of normal (*i.e.*, non-infectious) commercially purchased hamster plasma was addressed. Spiking experiments with normal plasma were then investigated to determine the extent of plasma inhibition on the PMCA assay. Thereafter two trials were performed with the optimized plasma-specific PMCA protocol to determine its' usefulness in discriminating between plasma from clinically-ill and negative control hamsters.

4.2 Results and Discussion

4.2.1 *Detection of prion protein by Western blot in the presence of plasma*

To apply the optimized PMCA assay to plasma, certain challenges associated with amplification and detection (*i.e.*, WB) of PrP^{Sc} in blood and/or its' components needed to be examined (41,139,201). Western blotting was used to determine whether PrP^{Sc} could be detected in NBH suspended in 10% normal hamster plasma (Figure 4.1). A protease resistant band, slightly smaller in size than PrP^{Sc} (~25-27 kD), was observed in all samples containing 10% plasma suggesting that a molecule within these preparations cross-reacted with primary (*i.e.*, 3F4) or the secondary antibody used in the WB. The protease-resistant band observed in the WB with plasma present was not a component of the NBH preparation, but rather a cross reactive epitope associated with the presence of plasma (Figure 4.2). The 25-27 kD protease-resistant band was also observed in 10% plasma samples diluted in water or PBS (*i.e.*, no NBH present) by WB (Figure 4.2). The intensity of this particular band was always greater in samples treated with PK (Figure 4.2), suggesting that that the cross-reactive epitope may be exposed during proteolytic degradation of a plasma-specific molecule.

To determine whether cross-reactivity was a function of the primary or secondary antibodies, two other monoclonal primary antibodies known to react with hamster PrP (SAF32 and SAF84), were tested in WB applications. Additionally detection with the secondary antibody (horse radish peroxidase, goat anti-mouse) only (*i.e.*, omitting the primary antibody) was investigated. Weak cross-reactive bands were observed when blots were probed with all three PrP

monoclonal antibodies when plasma was present in the sample (Figure 4.3). The appearance of this protease resistant band when the primary antibody was absent confirmed that the cross reaction was due to non-specific binding of the secondary antibody to a component of plasma during WB immunodetection. The smaller sized band could be easily distinguished from PrP^{Sc} in PMCA experiments (see subsequent section). The data also suggested that the PK digestion conditions were sufficient to resolve PrP^{Sc} from PrP^{C} in samples containing NBH with 10% plasma present.

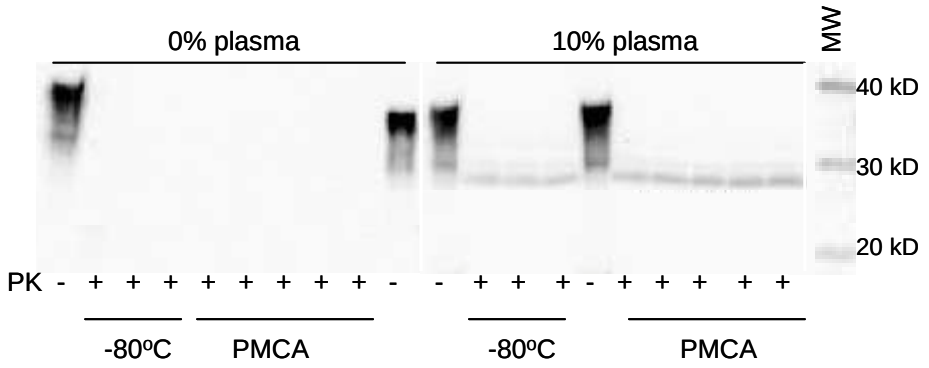

Figure 4.1 Western blot demonstrating a cross-reactive epitope associated with the addition of 10% normal plasma to normal brain homogenate (NBH) after PK digestion. Three independent replicates of -80°C controls and five independent replicates of PMCA samples were performed. Samples were treated with (+) and without (-) PK (200 µg/mL) digestion. Lane labeled 'MW' represents the molecular weight markers at 40, 30, 20 kD. Note the molecular weight of protease resistant band with 10% plasma present in NBH is ~25-27 kD, smaller than that of PrP^{Sc}.

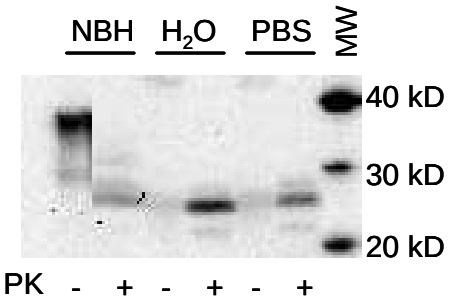

Figure 4.2 Western blot demonstrating the cross-reactive epitope (~25-27 kD) associated with 10% plasma present in NBH, water (H_2O) and PBS. Samples were treated with (+) and without (-) Proteinase K (PK) (200 µg/mL) digestion. Lane labeled 'MW' represents the molecular weight markers at 40, 30, 20 kD.

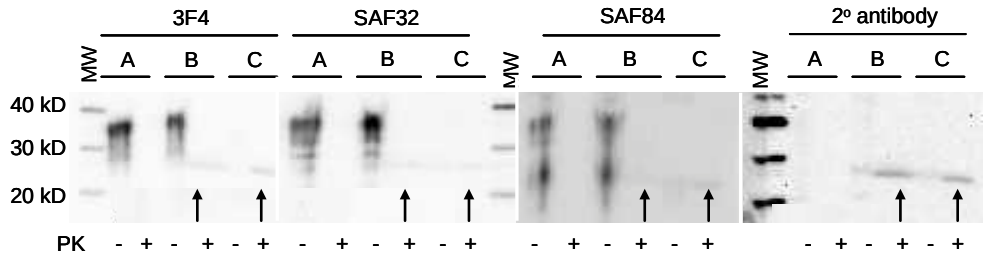

Figure 4.3 Western blot demonstrating the cross-reactive epitope associated with the addition of plasma (10%) is associated with non-specific binding of the secondary antibody. (A) Normal brain homogenate (NBH) only (*i.e.*, 0% plasma), (B) 10% plasma in NBH (volume/volume), (C) 10% plasma in PBS (volume/volume). Samples were treated with (+) and without (-) Proteinase K (PK) (200 µg/mL) digestion. Primary antibodies included 3F4, SAF32, and SAF84. The secondary antibody used for recognition was goat anti-mouse horse-radish peroxidase (HRP). Lanes labeled 'MW' represents the molecular weight markers at 40, 30, 20 kD. Arrows represent the samples where the cross-reactive epitope was visualized.

4.2.2 *Dynamic range of sensitivity of optimized PMCA assay in presence of plasma*

The dynamic range of sensitivity of the optimized PMCA assay (Chapter 3) in the presence of plasma was tested by spiking 263K hamster scrapie into hamster NBH supplemented with normal commercially purchased hamster plasma. In this set of experiments, conversion buffer (CB) was prepared with and without normal plasma present, which was then used to homogenize normal hamster brains (*i.e.,* preparation of NBH) for PMCA. Experimental dilutions series were performed similar to those described in the optimized PMCA protocol, with NBH used as the substrate (with or without plasma present) and hamster IBH as the PrP^{Sc} seed.

Surprisingly, and contrary to what had been described in the literature (41), the presence of normal hamster plasma greatly enhanced amplification of PrP^{Sc} by PMCA (~125x, Figure 4.4). This discrepancy may be attributed to one or several novel experimental approaches in this thesis. First, in order to avoid discrepancies associated with species barriers, the addition of normal plasma to the PMCA reaction was matched to the brain and seed substrate (*i.e.,* plasma, NBH and IBH were all derived from hamster). This was deemed necessary based on a trial experiment where normal plasma (commercially purchased) from other host animals (*i.e.,* xeno-specific plasma) was substituted for hamster plasma in the PMCA reaction while maintaining both normal and infectious brain homogenates from hamster. Indeed all of the xeno-specific normal plasma tested (*i.e.,* mouse, sheep, cow) inhibited amplification of hamster PrP^{Sc} in the PMCA reaction (Figure 4.5). Second, observations of PMCA inhibition with tissues containing

small amounts of plasma and/or serum (41), may not be entirely the result of plasma, but may be imparted by the test tissue or a combination of the test tissue with various blood components. Here, investigating PMCA under ideal conditions, where hamster brain PrP^{Sc} was serially diluted into hamster brain PrP^{C} the true impact on amplification due to the presence of plasma (normal) alone could be determined. The other potential contributor associated with the presence of plasma in the PMCA assay was the subsequent addition of the anticoagulant in which the blood sample was collected, in this case K_2EDTA. The effect of plasma on the PMCA reaction may vary for different anticoagulants and/or concentrations of anticoagulant used.

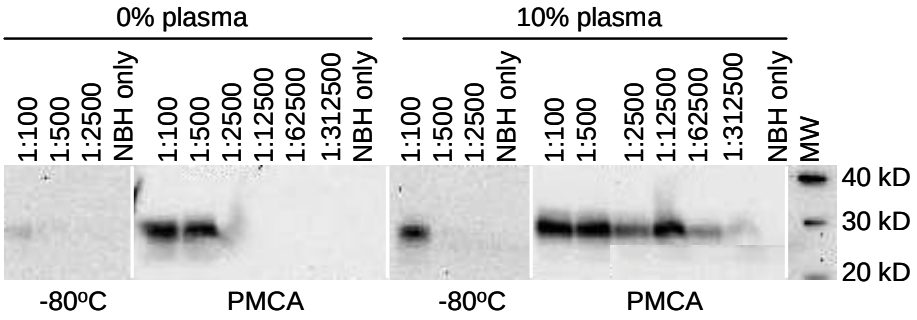

Figure 4.4 Western blot comparison of PrP^{Sc} amplified by PMCA with NBH prepared with (10%) and without (0%) the presence of normal hamster plasma. Infectious brain homogenate (IBH) was serially diluted (1:100-1:312500) into normal brain homogentate (NBH) and treated with Proteinase K (PK). Lane labeled 'MW' represents the molecular weight markers at 40, 30, 20 kD.

From published studies, the detection of PrP^{Sc} from plasma had been observed after 160 cycles of PMCA, with a starting concentration of 1% plasma (159), while a high degree (~50%) of inhibition in PMCA amplification was seen

in application to whole blood (230). Using the optimized PMCA assay the inhibition of plasma was examined through the addition of increasing normal hamster plasma concentrations to the PMCA assay (5, 10, 25, or 50%). The WB of one independent replicate is shown in Figure 4.6 and the amplification results of two to four independent replicates are tabulated in Table 4.1. A small stimulatory effect was observed when the control, CB and its' components were diluted with PBS (*i.e.,* PBS controls) rather than plasma (Table 4.1), however a much greater accentuation effect was always apparent when the PMCA assay was supplemented with hamster plasma.

In the 1^{o} round of PMCA, a concentration of 50% plasma was inhibitory to the PMCA reaction, while the addition of 10% plasma led to the highest average amplification (Table 4.1). Interestingly, after 4 rounds of sPMCA, where the previous round PMCA product was diluted 1:10 in NBH for all subsequent PMCA rounds, the accentuation effect produced by plasma dampened (*i.e.*, diluted out with subsequent rounds of PMCA) as determined by the eventual similar levels of average amplification observed in the 0% plasma control. Greater than 4.0 $\log_{10}$ of amplification was achieved in the presence of 5 - 25% plasma after just two 16 hour rounds (32 cycles) of PMCA.

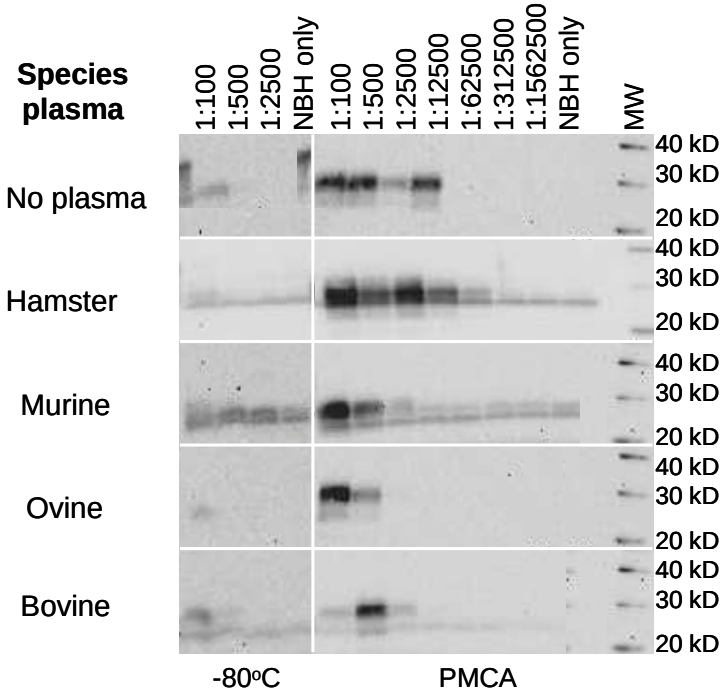

Figure 4.5 Western blot demonstrating PrP^{Sc} amplification after one round of PMCA performed in the presence or absence of normal plasma from different animal species. For samples in which animal plasma was present, a 10% concentration was used. Hamster nfectious brain homogenate (IBH) was serially diluted (1:100-1:1562500) into normal hamster brain homogentate (NBH) with normal (commercially purchased) plasma from hamster, mouse, sheep, or cow added. Samples were digested with Proteinase K (PK). Lane labeled 'MW' represents the molecular weight markers at 40, 30, 20 kD.

Table 4.1 Average amplification of PrP^{Sc} associated with sPMCA assay containing increasing concentrations of normal hamster plasma within the 1^{o} round of PMCA.

% normal plasma/PBS[a]	Average amplification[b]					
	1^{o} PMCA		2^{o} PMCA		3^{o} PMCA	4^{o} PMCA
	plasma	PBS	plasma	PBS	plasma	plasma
0%	~181x	5x	>6810x	250x	>31500x	~1575000x
5%	~1092x	125x	~31250x	1250x	ND[c]	ND
10%	>2025x	125x	>50250x	6250x	>157500x	>1625000x
25%	~1025x	25x	>56250x	1250x	>187500x	>1875000x
50%	~3x	ND	~6250x	ND	>37500x	>375000x

[a] plasma/PBS added in 1^{o} round only, with all subsequent rounds of sPMCA performed with NBH in conversion buffer (CB) only

[b] average amplification calculations were based on two to four replicates for plasma

[c] ND = no data

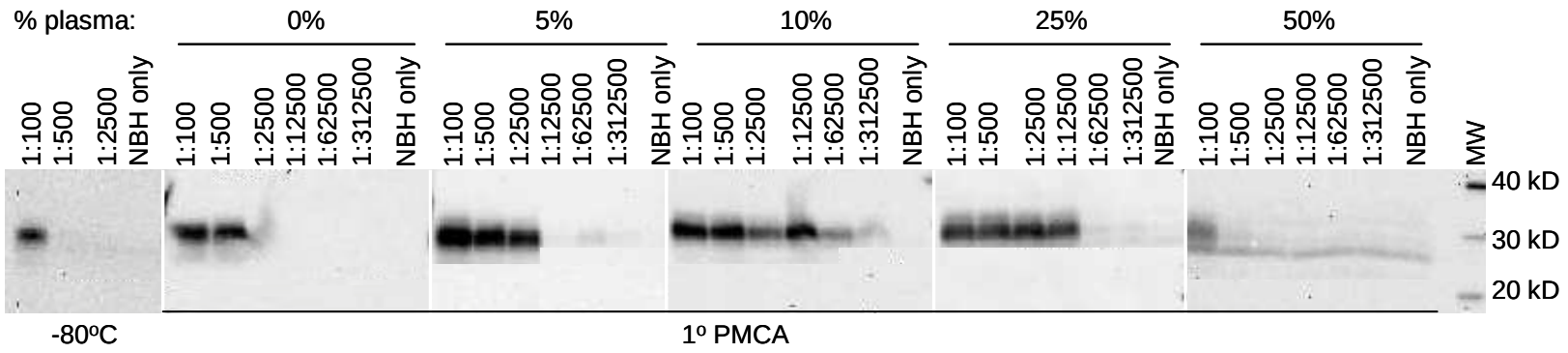

Figure 4.6 Western blots demonstrating the effect of increasing normal plasma concentration on the amplification of PrP^{Sc} by PMCA. Normal brain homogenate (NBH) samples were prepared with the addition of various concentrations of plasma (0, 5, 10, 25 and 50%) and the PMCA seeded with varying dilutions (1:100 – 1:312500) of infectious brain homogenate (IBH). Lane labeled 'MW' represents the molecular weight markers at 40, 30, 20 kD. Note: that the intensity of non-specific background band associated with binding of the secondary antibody to plasma intensified with increasing concentrations of plasma.

Although the addition of 50% plasma to the 1^{o} PMCA was inhibitory, sPMCA negated the inhibitory effects, and in fact, accentuated protein misfolding through subsequent rounds (Table 4.1). This effect was observed under optimized sPMCA conditions (Chapter 3), where the previous round product was diluted 1:10 into new NBH prior to performing the next round of PMCA. Therefore the 50% normal plasma present in the 1^{o} PMCA was reduced to 5% in the 2^{o} PMCA, 0.5% in the 3^{o} PMCA, etc. This implied that the addition of low levels of plasma to the PMCA reaction mixture in later or subsequent rounds of PMCA may increase PrP^{Sc} amplification further. To investigate this potential, the addition of normal plasma (1, 5 and 10%) in the NBH diluent for subsequent rounds of PMCA (2^{o} and 3^{o} PMCA) was performed (Table 4.2). The addition of 1% normal plasma in the 3^{o} PMCA resulted in little accentuation effect when normal plasma was present in the 1^{o} PMCA (10%) or not (0%). The addition of 5% or 10% normal plasma in the 2^{o} round of PMCA, however, led to >6.75 $\log_{10}$ of amplification after only two rounds of PMCA, clearly demonstrating the advantage associated with the presence of contiguous normal plasma in the diluent of the subsequent round of sPMCA.

Table 4.2 Effect of the concentration of normal hamster plasma on PrP^{Sc} amplification by sPMCA when added during different rounds of PMCA.

	1° PMCA		2° PMCA		3° PMCA	
	% plasma[a]	Amplification	% plasma[b]	Amplification	% plasma[b]	Amplification
Trial 1	0	125x	0	>1250x	0	12500x
					1	62500x
	10	3125x	0	>31250x	0	>312500x
					1	312500x
Trial 2	0	25x	0	1250x	0	500x
					1	2500x
	10	625x	0	>1250x	0	>12500x
					1	>12500x
Trial 3	0	25x	5	62500x	ND[c]	ND
			10	312500x	ND	ND
	10	125x	5	~7812500x	ND	ND
			10	~7812500x	ND	ND

[a] the % plasma added to conversion buffer (CB) used to prepare NBH for the 1° PMCA
[b] the % plasma added to the conversion buffer (CB) used to prepare the NBH for the diluent that the previous round PMCA product was diluted 1:10 into
[c] ND = no data

In an attempt to further increase the overall level of amplification of PrP^{Sc} in the presence of plasma, the duration of sonication during the 1° round of PMCA was manipulated (Table 4.3). Increasing the duration of the 1° PMCA resulted in greater amplification of PrP^{Sc} in both the presence (10%) and absence (0%) of normal plasma. The data observed in the absence of plasma (0% plasma) appeared to contradict the data presented in Chapter 3 (Table 3.4) where increasing the length of the first round of PMCA had little effect on increasing the amplification of PrP^{Sc}. However, a major difference from the initial experiments in Chapter 3 was the use of PBS as the control diluent in the 1° PMCA of these experiments (*i.e.*, 10% PBS was added to the NBH to control for the dilution of the NBH with plasma). This suggested, as was previously shown (Table 4.1), that dilution of the CB contributed to a slight increase in the levels of PrP^{Sc}

amplification. Nevertheless, the presence of plasma further accentuated the amplification of PrP^{Sc} across all time points measured (Table 4.3).

One 48 hour round of PMCA (*i.e.,* 96 cycles) in the presence of 10% normal hamster plasma produced ~4.5 $\log_{10}$ of PrP^{Sc} amplification (Figure 4.7). This result was greater than the 6600x (Table 3.1) amplification of PrP^{Sc} (140 PMCA cycles) reported by Castilla *et al*. (42). Likewise, 2 rounds of amplification with either 16 hours, 24 hours, or 48 hours led to ~6.75 $\log_{10}$ amplification of PrP^{Sc} by PMCA in the presence of contiguous plasma; an overall level of amplification similar to that observed by Castilla *et al*. (42) in which they reported a 10 million fold (7 $\log_{10}$) amplification after 2^{o} PMCA. A major difference in this thesis, was the observation that only 64 cycles of PMCA (two 16 hour rounds) in normal hamster plasma was equally as sensitive at detecting of PrP^{Sc} from IBH as 214 cycles of PMCA by Castilla *et al*. (42) in hamster brain homogenates alone. Based on fact that Castilla *et al*. (42) amplification levels were sufficient for the detection of PrP^{Sc} in the buffy coat fraction from 16 of 18 (89% sensitivity) infected and clinically-ill hamster samples after six rounds of PMCA (42), suggested that the plasma-specific assay developed in this thesis may be more sensitive at detection of PrP^{Sc} in infected animals.

An extremely interesting finding was the observation that after two 48 hour rounds (96 cycles) of PMCA in the presence of contiguous plasma, a protease-resistant band of the same molecular weight as PrP^{Sc} was observed in the 'NBH only' sample in this thesis work (Figure 4.7). After two rounds of 16 hour (64 cycles) sPMCA with contiguous plasma, no protease-resistant PrP^{Sc} band was

ever observed in the NBH control in five independent WB replicates. However, after 3 rounds of 16 hour sPMCA (96 cycles) with contiguous plasma present, a protease resistant PrP^{Sc} band was detected in the NBH control from 1 of 2 independent experiments (Figure 4.8). It is important to note that a protease-resistant band was never observed in samples that did not contain plasma, under any of the conditions investigated.

The data suggests possible '*de novo*' synthesis of PrP^{Sc} within samples subjected to PMCA in the presence of plasma. Arguably, the appearance of the protease-resistant band in these samples may not represent spontaneous synthesis of PrP^{Sc} *in vitro,* but rather extremely small quantities of misfolded prion protein that may naturally circulate in plasma or normal brains of hamsters, but for which inherent biological mechanisms prevent the propagation of these misfolded proteins *in vivo* (261). Interestingly similar *de novo* generation of PrP^{Sc} has been reported by Deleault *et al*. (58) using purified substrates for sPMCA. They found that after as few as 4 rounds of 24 hour/round of sPMCA (192 cycles) when purified PrP^{C} was supplemented with a synthetic polyanion (*i.e.*, poly A), *de novo* PrP^{Sc} appeared. Thorne and Terry (237), confirmed that the presence of poly A in the reaction mixture of PMCA led to *de novo* PrP^{Sc} after three (144 cycles) or four (192 cycles) rounds of sPMCA in uninfected blood leukocyte samples. In this study *de novo* PrP^{Sc} was never detected if two or less rounds of sPMCA (<96 cycles) were performed. It could be reasoned that maintaining contiguous plasma in subsequent rounds of sPMCA, as presented here, decreased the number of cycles required to generate *de novo* PrP^{Sc}. The potential that the *de novo* PrP^{Sc}

detected in this thesis work was associated with laboratory contamination is unlikely based on similar results of experiments in a prion-free facility where no infectious material was utilized (Appendix A.1). For simplicity, the term *de novo* PrP^{Sc} will be used for the remainder of this thesis to refer to instances where a protease resistant band, presumably PrP^{Sc}, was detected by WB in the absence of an infectious seed.

Table 4.3 Effect of the duration of PMCA on the amplification of PrP^{Sc} in the presence (10%) or absence (0%) of normal hamster plasma.

Duration 1° PMCA	Average amplification[a]	
	0% plasma	10% plasma
16 hours	~65x	>375x
24 hours	>15x	>625x
48 hours	>625x	>46875x

[a] based on two independent replicates

4.2.3 *Application of the PMCA assay for prion detection in plasma samples from scrapie infected 263K hamsters*

To validate the potential application of the modified, 'plasma-specific PMCA' to detect 263K scrapie infected hamsters, plasma samples were collected and prepared from six infected, clinically-ill and six non-infected control hamsters. Plasma from both the infected and non-infected animals was diluted to a starting concentration of 10% in NBH prepared with CB (*i.e.*, no normal plasma present). Serial PMCA was performed on the samples in two independent trials on two different PMCA sonicators (Table 4.4). The primary round of PMCA contained the test plasma (collected from infected or non-infected animals), and all subsequent rounds of the sPMCA were performed in the presence of either 10% normal hamster plasma or with no plasma addition.

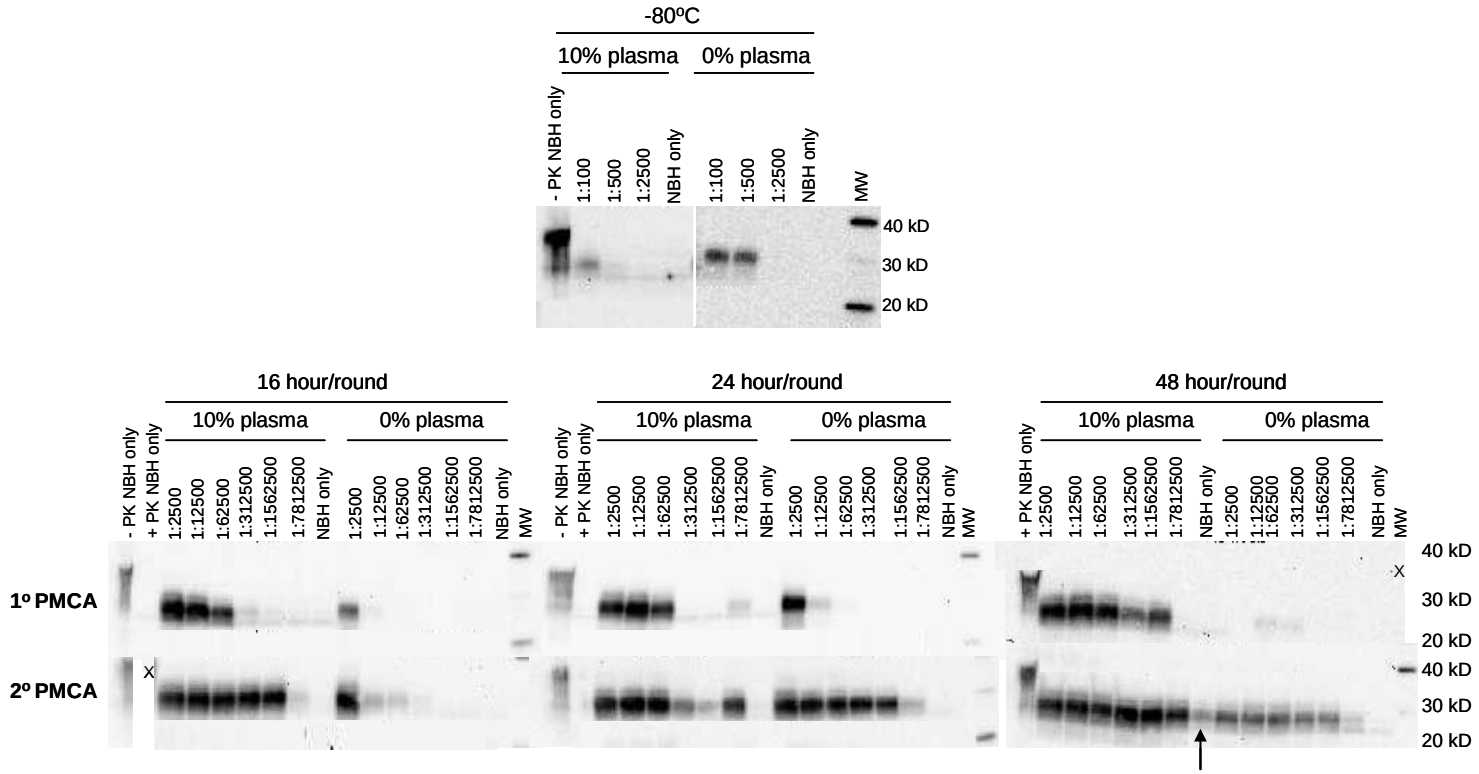

Figure 4.7 Western blots demonstrating the effect of the presence of hamster plasma and duration of PMCA on amplification of PrP^{Sc} by sPMCA. 1^{o} PMCA was carried out in the presence (10%) or absence (0%) of normal plasma (infectious brain homogenate [IBH] was serially diluted [1:100-17812500] into normal brain homogenate [NBH] with normal plasma present) for 16, 24, 48 hrs, followed by dilution (1:10) of the 1^{o} PMCA product into new NBH with contiguous normal plasma (5%) for an additional round (2^{o} PMCA) of 16, 24, 48 hrs of PMCA. Lanes labeled 'MW' represents the molecular weight markers at 40, 30, 20 kD. Lanes labeled with an 'X' represent no sample. Arrow (↑) represents the control sample (NBH only) in which a protease-resistant band appeared after two 48 hr rounds of sPMCA. Western blot of non-amplified controls (-80ºC with no sonication) containing 10% plasma or no plasma are shown in the upper panel.

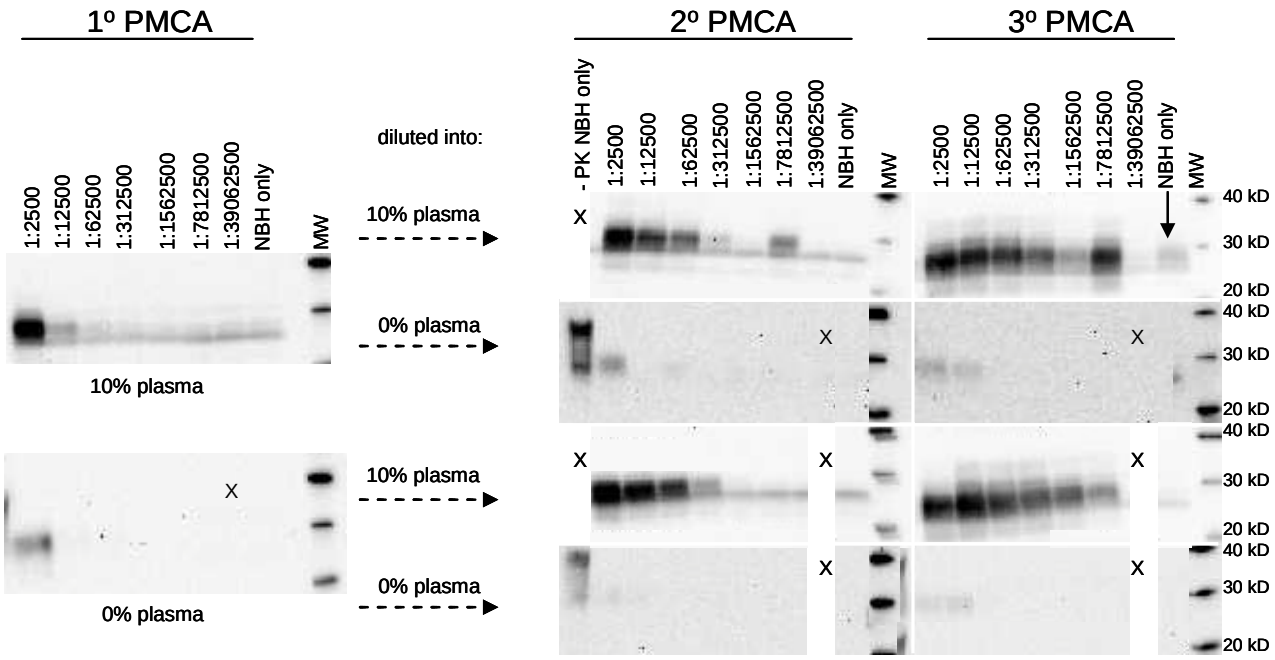

Figure 4.8 Western blots demonstrating *de novo* synthesis of PrP^{Sc} after three rounds of sPMCA. 1º PMCA was run in the presence (10%) or absence (0%) of normal hamster plasma (infectious brain homogenate [IBH] was serially diluted [1:100-1:39062500] into normal brain homogenate [NBH] with normal plasma present) for 16 hrs. 1º and 2º PMCA products were diluted into normal brain homogenate (NBH) with contiguous normal plasma present (10%) or absent (0% plasma) for the following rounds of PMCA. Lanes labeled 'MW' represents the molecular weight markers at 40, 30, 20 kD. Lanes labeled with an 'X' represent no sample. Solid arrow (↓) represents the control sample (NBH only) in which a protease-resistant band appeared after three 16 hr rounds of sPMCA (96 cycles) in the presence of contiguous plasma.

In the first trial (Machine A), 2 out of 5 PrP^{Sc} reactive bands were detected by WB after the 5^{th} round of PMCA in the infected hamster plasma samples where contiguous plasma was maintained after the 1^{o} PMCA (Table 4.4). By the 7^{th} round of PMCA a total of 3 of the 5 infected hamster plasma samples displayed the presence of PrP^{Sc}, again only when contiguous normal plasma was present in sPMCA. By the 7^{th} PMCA round two of the non-infected plasma controls displayed a PrP^{Sc} reactive band as well suggesting the possible *de novo* synthesis of PrP^{Sc} in the presence of non-infected hamster plasma. In the second trial (Machine B), a PrP^{Sc} reactive band was detected by WB in all infected hamster plasma samples after the 6^{o} PMCA using the plasma-specific PMCA. In this trial, as early as the 4^{o} PMCA non-infected control samples displayed a PrP^{Sc} band in the presence of contiguous plasma (Table 4.4), again suggesting the possibility of *de novo* PrP^{Sc} synthesis in the presence of plasma. In the absence of the plasma (0% plasma) the level of amplification achieved was unable to distinguish prions in plasma from infected and non-infected hamsters (Table 4.4).

Evaluation of the PMCA control samples (commercially purchased normal hamster plasma), performed in parallel to the two trials, revealed that in samples where NBH was the only brain constituent present (*i.e.*, controls in which no IBH was ever used as an PrP^{Sc} seed) that *de novo* PrP^{Sc} was detected as early as the 4^{o} round of sPMCA and eventually in all NBH controls where contiguous normal plasma was maintained in sPMCA. In conditions in which plasma (10%) was present only in the 1^{o} PMCA round, and where plasma was not supplemented throughout sPMCA, the appearance of *de novo* PrP^{Sc} was never observed.

Although maintaining contiguous plasma throughout sPMCA complicated the interpretation of the results, the use of the optimized PMCA protocol (Chapter 3) alone was not sensitive enough to differentiate between infected and non-infected hamster plasma samples. Unfortunately the plasma-specific PMCA was also unable to distinguish between plasma samples of infected and non-infected hamsters under the current conditions. Further modification and/or manipulation of the plasma-specific PMCA may increase its potential for successful application to plasma from diseased animals.

Table 4.4 Application of sPMCA to detection of PrP^{Sc} in plasma samples collected from normal (non-infected) and clinically-ill (infected) hamsters infected with 263K scrapie.

	Trial #1 Machine A				**Trial #2 Machine B**			
	Plasma from infected hamsters		**Plasma from non-infected hamsters**		**Plasma from infected hamsters**		**Plasma from non-infected hamsters**	
	% plasma present in subsequent rounds of PMCA[a]							
	0%	**10%**	**0%**	**10%**	**0%**	**10%**	**0%**	**10%**
PMCA round	**number of PrP^{Sc} positive samples / total samples tested by PMCA**							
2°	0/6	0/6	0/6	0/6	0/6	0/6	0/6	0/6
3°	0/6	0/6	0/6	0/6	0/6	0/6	0/6	0/6
4°	0/6	0/6	0/6	0/6	ND[b]	0/6	ND	1/6
5°	0/6	2/5	0/6	0/6	ND	5/6	ND	4/5
6°	0/6	3/5	0/6	0/6	0/6	6/6	0/6	5/5
7°	0/6	3/5	0/6	2/6	ND	ND	ND	ND

[a] the % plasma is the amount of normal plasma added in each round of PMCA
[b] ND = no data

The findings associated with the addition of plasma to the PMCA assay have indicated a knowledge gap that has not been previously addressed in relation to plasma. The potential that plasma provides a co-factor of the PMCA reaction thereby accentuating *in vitro* conversion of PrP^{C} to PrP^{Sc} is an extremely

important observation and may lead to the identification of additional accessory factors required for prion propagation. The generation of a protease resistant band, referred to here as *de novo* PrP^{Sc}, in the lack of an infectious seed in the presence of plasma is also noteworthy and discussed in more detail in Chapter 5 of this thesis.

5 GENERAL DISCUSSION

The importance of an ultrasensitive, reliable ante-mortem detection method for diagnosis of prion diseases has become increasingly more apparent in the past 15 years as public safety concerns arise about the contamination of food supplies, medical procedures and blood/organ donations. Moreover, increasing evidence of transmission of prions through exposure to bodily fluids and wastes in animal prion diseases emphasizes the need to develop tools to diagnose prion-infected animals early during infection. Confirmed knowledge of environmental soil and water contamination also poses an enormous challenge in the control and prevention of these diseases, necessitating the application of these tools to detection of prions in environmental matrices.

Few prion detection methodologies are capable of detecting low level or asymptomatic carriers of prion disease, and their usefulness has been restricted to primarily confirmatory diagnosis post-mortem (264). In the past decade several *in vitro* assays have been developed in an attempt to advance more sensitive ante-mortem diagnostic tools. These tools have focused specifically on either increasing sensitivity of detection of PrP^{Sc} or identifying other ante-mortem markers of infectious TSEs (*i.e.*, circulating nucleic acid, FIAsH fluorescence) (48,91).

The PMCA assay mimics the pathological conversion of PrP^{C} to PrP^{Sc} significantly shortening the time needed for analysis compared to *in vivo* infectivity methods (days compared to years in an *in vivo* system), and for which the amplified end product has been confirmed as infectious (40). Over the last

few years, the PMCA assay has been shown to be an incredibly sensitive blood-based assay, capable of pre-symptomatic disease detection in animals (202). Since the inception of this thesis, PMCA has been adapted to increase low level detection of misfolded prions in various animal species and from various tissues, fluids and complex matrices (123,159,163,213,220). Thus, PMCA has emerged as a potentially versatile ante-mortem tool for enhanced detection of prions.

The PMCA assay has, however, only recently become a commonly used prion research tool. Between 2001, when the assay was first described, and 2006 when this thesis was initiated, a collective number of 16 publications originating from only 4 independent research groups had reported adapting PMCA for amplification of prions *in vitro*. Following proof of the concept of PMCA by Saborio *et al*. (204), Soto and colleagues further utilized the assay to confirm infectivity (although reduced) of the PMCA product (40), detect PrP^{Sc} in the buffy coat fraction of blood (42), detect PrP^{Sc} pre-symptomatically in brain homogenates (202) and blood (202), and develop an automated PMCA protocol (203). Two of the three other independent laboratories research focused on non-denaturing PMCA (125,143) and explored the direct effect of various components on the PMCA reaction; stimulatory effects with RNA (60), polysynthetic anions (57) and manganese (125), and an inhibitory effect with copper (170). The third independent group, utilizing conventional PMCA, determined the assay was inhibited with the use of recombinant rather than hamster PrP^{C} (21), that a minimum PrP^{Sc} seed was necessary for successful amplification (182), that efficient PrP^{Sc} conversion was possible with indirect sonication (210), and that

the reduction of infectivity of the PMCA product, previously reported by Castilla *et al.* (40), was due to smaller PrP^{Sc} aggregation size that delayed disease onset (250). Although conventional manual PMCA, utilizing the 263K hamster model, had been established and successfully utilized by the Kretzschmar laboratory (accounting for all four publications on conventional PMCA outside of Soto and colleagues up until 2006), the level of amplification achieved by Saborio *et al*, (204) was never replicated (Table 3.1). Sarafoff *et al.* (210) believed that although the PMCA reaction was an efficient method to generate PrP^{Sc} *in vitro* that not all factors determining amplification efficiency were well understood or characterized.

For this reason, a major focus of the research in this thesis was the development and optimization of the PMCA assay using the most widely studied animal prion model, 263K hamster scrapie. To address the discrepancies amongst laboratories in amplification rates reported (6,21,134,210) all PMCA methods in the published literature were compared and from this information a 'basic protocol' was developed. The basic protocol described in this thesis was based primarily on the work of Castilla *et al.* (37,41), but with slight modifications from other published studies (Table 3.1). Employing the basic protocol alone resulted in low but consistent and reproducible amplification of PrP^{Sc} *in vitro* (*i.e.*, ~5x amplification after 24 hours, 48 cycles). This level of amplification was considerably lower than 6600x observed by Castilla *et al.* (42) after 72 hours (144 cycles). Variations in the published PMCA methodologies were reviewed (Table 3.2 and 3.3) and divided into internal variables (*i.e.*, duration of PMCA, pre-

incubation of samples prior to PMCA, requirement of sonication, sonication intensity, incubation temperature, sample vessels used) and external variables (*i.e.*, requirement of daily CB preparation, detergent concentration, necessity of normal brain perfusion, fresh or frozen NBH for PMCA, effect of freeze-thawing IBH). These variables were systematically modified in order to develop an optimized PMCA assay for 263K scrapie. The focus during the development of the optimized PMCA relied heavily on maintaining consistent and reproducible amplification, while assessing whether these key variables and/or the application of sPMCA would lead to a positive or negative effect on overall levels of PrP^{Sc} amplification.

Several publications had reported a clear relationship between the level of amplification and number of cycles used in PMCA (37). Saborio *et al.* (204), observed exponential increases in amplification with increasing rounds with manual PMCA and this was reaffirmed by Saa *et al.* (203) for the automated PMCA system. The basic PMCA protocol of this thesis focused on a single 24 hour round of PMCA. Extending the duration of PMCA (up to 48 hours) did not enhance overall levels of PrP^{Sc} amplification. This result was similar to observations by Sarafoff *et al.* (210) and Zukas *et al.* (267) who both reported that cycles of incubation and sonication increased amplification only to a point, after which additional PMCA cycles resulted in an adverse effect. A time course analysis performed during the optimization of PMCA of this thesis revealed that similar and detectable levels of PrP^{Sc} amplification observed with the basic protocol (24 hours) could be replicated with as few as 3 hours of PMCA. A

reduction in the duration of the basic protocol from 24 hours (48 cycles) to 16 hours (32 cycles) resulted in the most consistent and improved amplification (~25-125x amplification) for all durations tested during optimization of the PMCA assay.

Although one study had demonstrated that as much as 6x amplification of PrP^{Sc} in the absence of sonication with non-denaturing PMCA (226), sonication was observed to be a key requirement and necessary to achieve amplification in this thesis. The possibility of obtaining amplification with shaking instead of sonication between periods of incubation (*i.e.,* QuIC), known to result in efficient PrP^{Sc} amplification (7), was not tested. Quaking induced conversion, is a simplified *in vitro* technique that utilizes recombinant (rPrP) as the conversion substrate for PrP^{Sc}. The diagnostic potential of this assay has been clearly demonstrated in its' ability to detect PrP^{Sc} in cerebrospinal fluid and olfactory mucosa (7,20).

Evaluation of the sonication intensity reported in the literature recommended maximal or near maximal power settings (8-10) to achieve amplification for 263K hamster (41,201,220). More consistent amplification was obtained at a power setting of 6. There are several possible explanations for reduced consistency in PMCA amplification at higher power settings (>6). It is possible that more intense sonication may damage critical co-factors found in NBH preparations. It is widely recognized that crude brain homogenates not only provide a source of PrP^{C} as a substrate for template misfolding during PMCA, but also provide the necessary co-factors needed for misfolding of PrP^{C} into PrP^{Sc}

(205,226). Alternatively, it is known that PrP^{Sc} conversion requires a minimum sized infectious seed in order to initiate template directed misfolding (182). More intense sonication conditions may disrupt PrP^{Sc} aggregates into particles too small to efficiently act as a seed for further template directed misfolding. It was also observed that more intense sonication led to dispersion of sample in the tube, and in some cases the contents in the tube did not come back into contact for further conversion. Trials with a lower power setting of 4 led to considerably less amplification than power setting 6. The optimized PMCA was thus adjusted to a power setting of 6.

Detrimental effects on PMCA amplification had been reported with longer time frames (>75 hours) presumably associated with extended incubation of the substrate at 37^{o}C (41), and possibly disaggregation of infectious seeds (as described above). However, other studies have reported successful amplification of PrP^{Sc} at both 25^{o}C and 37^{o}C with continuous shaking (143), and as high as 45^{o}C - 65^{o}C when shaking was substituted for sonication bursts (7). Attempts to maintain the incubation temperature at 37^{o}C during optimization of the PMCA protocol here did not result in increased levels of amplification. In fact, it appeared that PrP^{Sc} amplification may be increasing with an elevation in temperature, similar to the observations with QuIC (7).

Investigation into the effect of slight changes in detergent concentration (*i.e.,* Triton-X 100) in the CB preparation for the basic protocol (0.9-1.1%) did not change the level of PrP^{Sc} amplified by PMCA. Studies in other species (deer and mice) have demonstrated that small additions (0.05%) of other detergents,

specifically saponin or digitonin, were required for efficient amplification (134,160). The effectiveness of these detergents were not examined in this thesis work and have yet to be documented in the literature as necessary for the 263K hamster species. These detergents have, however, been shown to solubilize 'PrP^{Cres}', a PrP^{C} aggregate that forms during PMCA (160,162). The presence of PrP^{C} aggregates and/or lack of an additional or appropriate detergent concentration may partially explain the low levels of amplification achieved during the PMCA optimization.

The difficulty with PMCA reproducibility in many laboratories has also been linked to brain harvest conditions (267). The necessity of a good quality substrate (PrP^{C}), that is brains perfused at the time of sacrifice to remove blood and its' components, has been regarded as essential to achieving amplification with the PMCA assay (37,41,201). Contrary to this, during optimization of the basic protocol similar levels of amplification were achieved with and without perfusion of the normal hamster brain used for NBH preparation. This implied that blood and/or some of its components may not be inhibitory to the PMCA reaction, however the use of perfused normal brains was maintained in the optimized protocol to avoid confusion when addressing the effect of plasma to the PMCA reaction.

Lastly in the optimization portion of this thesis the application of sPMCA to the basic protocol was investigated. Similar to what had been reported in the literature elevated levels of amplification were achieved with additional rounds of PMCA (21,41,42,134,182,202,203,220,251). The basic protocol adopted 2

rounds of sPMCA (each consisting of 16 hours) with a 1:10 dilution of the previous round PMCA product into fresh NBH, achieving ~3 $\log_{10}$ of PrP^{Sc} amplification after only 32 hours (64 cycles). This level of amplification was similar to that presented by Castilla *et al.* (42) after 1 round of 72 hours (140 cycles), however 2 rounds (107 hours, 214 cycles) of PMCA by Castilla *et al.* (42) led to much greater amplification (7 $\log_{10}$ amplification). Additional sPMCA did not greatly enhance PrP^{Sc} amplification of the basic protocol in this thesis.

With optimization of the basic PMCA protocol completed, the second objective of this thesis, the adaptation and application of the PMCA assay to detect prions in plasma was addressed. Modifications to the WB procedure (*i.e.,* one step WB) and/or detection methodologies that do not require proteolytic degradation (*i.e.,* CDI, SOFIA) of the sample have been deemed necessary for plasma samples in several studies (34,94,104,120,139,159,199). When the efficiency of the WB in the presence of normal hamster plasma (commercially purchased) was tested in this thesis a cross-reactive band (~25-27 kD) was observed. The cross-reactive band was found to be associated specifically with plasma and not the NBH preparation. The intensity of the cross-reactive epitope increased after digestion with PK and was proven to be an artifact of non-specific secondary antibody binding to a component of plasma. The non-specific band was distinguishable from PrP^{Sc} detected by differences in molecular weight.

Blood preparation protocols and concentration of prions from other bodily fluids (*i.e.,* urine) have led to complications in the detection of PrP^{Sc} (77,90,159,262). Blood, or components thereof, have also been associated with

negative effects on PMCA amplification (41,201). Therefore no upfront processing was carried out on normal plasma in this thesis work. Contrary to work described in the literature, the presence of 10% normal hamster plasma in the NBH substrate used in PMCA led to an accentuation of PrP^{Sc} amplification with the optimized conditions outlined in this thesis. Thus, it appeared that an accessory factor was present in normal plasma, and when added to the NBH preparation, aided in the conversion of PrP^{C} to PrP^{Sc} during PMCA. Interestingly an inhibitory effect on hamster PrP^{Sc} amplification was exhibited when xeno-specific normal plasma from three other species (*i.e.*, mouse, sheep, cow) were tested, suggesting that the accentuating effect of this accessory co-factor on protein misfolding may be species specific.

The observation that a potential species-specific co-factor in plasma can accentuate prion propagation is a very important finding of this thesis and has not been documented in the scientific literature. This finding stems from unique approaches utilized in this thesis. In order to address the inhibitory nature of plasma in the context of this thesis, normal hamster plasma was added to the optimized PMCA assay, and for which a hamster-derived substrate (PrP^{C}) and seed (PrP^{Sc}) for PMCA was combined. Although all other studies with plasma also utilize a species matched PrP^{C} source, they have relied on the infectious seed (PrP^{Sc}) to originate from the plasma added to the PMCA reaction. The low concentration (1%) of plasma tested by Murayama *et al.* (159) may not have been a high enough concentration to observe an accentuation effect on PMCA, as was observed in this thesis. Similarly, the high concentration of plasma used by

Rubenstein *et al.* (199) (*i.e.*, 500 µL of plasma with 100 µL NBH) was likely too high to exhibit an accentuating effect, as was observed in the present thesis.

Until very recently (May 2010) no other published work had evaluated the effects on amplification of PrP^{Sc} spiked with a specific component of blood, as had been investigated in this thesis (230). When this approach was used, only a 1% concentration of whole blood was added to the PMCA starting material, which resulted in ~50% reduction in amplification capability. This suggests that the protein-misfolding accentuation factor(s) associated with plasma may be inhibited, blocked or outcompeted for when present in conjunction with whole blood. This is not unexpected as it has been found that other tissues contaminated with whole blood also inhibit the PMCA reaction (41). The infrequency of symptomatic prion disease occurrence is further evidence that the body must have one or several control mechanisms to limit blood borne co-factors that trigger or enhance PrP^{Sc} conversion *in vivo*.

The accentuation effect produced with plasma may potentially be the result of a direct or indirect association with the anticoagulant (and its' concentration) in which the blood sample was originally collected. In this thesis, the anticoagulant K_2EDTA was used. The anticoagulant sodium citrate and/or EDTA was used by Castilla *et al.* (41) and Thorne and Terry (237), and successful amplification with PMCA in buffy coat and blood leukocyte preparations was observed, while sodium citrate alone was used in the Rubenstein *et al.* (199) study to detect PrP^{Sc} in blood plasma. Both of these anticoagulants bind calcium ions to inhibit clotting. Heparin, utilized by Murayama *et al.* (159) in their work on

PMCA, acts on antithrombin to inhibit blood clotting. Interestingly the study performed with heparin has been the only study capable of detecting PrP^{Sc} with a conventional detection method (*i.e.,* WB) after sPMCA (159) and heparin sulfate has been implicated in biogenesis of PrP^{Sc} in cell culture (18,110).

The importance of a cellular co-factor(s) triggering, enhancing or accelerating prion formation is not a recent finding. Evidence over the last 20 years has supported the fact that a cellular factor may be associated with PrP^{Sc} and that may be associated and/or responsible for disease outcomes (23,73). The discovery that *in vitro* conversion of PrP^{C} to PrP^{Sc} is extremely limited with a purified substrate and seed, exemplifies that a component(s) of the crude brain homogenate is necessary and supports the cellular factor requirement for *in vivo* misfolding. Numerous molecules have been found to interact with PrP^{C}, which include both protein and non-proteinaceous factors (*i.e.,* glycoaminoglycans), which may or may not have an effect on PrP^{Sc} propagation. Examples include plasminogen that co-precipitates and selectively binds PrP^{Sc} (74) and heparin sulfate that is a required receptor and co-factor in PrP^{Sc} formation and prion replication in tissue culture (18,110). Plasminogen association with PrP^{Sc} has since been disputed as related to detergent conditions and not a result of components in blood, as determined by Shaked *et al.* (214). Evaluation of necessary cellular factor(s) for prion association and/or propagation is extremely reliant on the technique used. *In vitro* conversion techniques offer the clear advantage of monitoring PrP^{Sc} conversion and increases or decreases in PrP^{Sc}

amplification with the addition of a cellular co-factor(s) are likely associated with prion propagation.

Co-factors known to enhance prion misfolding using non-denaturing PMCA include specific RNA molecules (poly A) and molecules with a free sulfhydryl group that are believed to be essential during conformational change of PrP^{C} to PrP^{Sc} (60,143). Subsequent investigation into the stimulatory effect of RNA molecules has revealed that there are species specific differences in co-factor utilization (59). Very recently Abid *et al.* (1) have investigated this extensively, reporting that the conversion co-factor is not a nucleic acid, protein or small chemical compound (salt, ions or other chemical that is <10 kDa). They believe that the co-factor may cooperate with other molecules or several non-specific molecules since various classes of molecules (*i.e.*, poly A synthetic RNA, bovine serum albumin, heparin and fatty acid mixtures) were able to induce conversion, but not to the same degree as crude brain homogenates. The study also postulated that the conversion factor is not species specific, since substitution of other mammalian brain homogenate substrates (*i.e.*, knock-out mouse, rabbit) still resulted in hamster PrP^{Sc} amplification, nor is the co-factor exclusive to the brain, as various rabbit organs (*i.e.*, liver, kidney, heart, muscle) used as PrP^{C} substrate were capable of efficient hamster PrP^{Sc} conversion. The finding that plasma accentuation is species-specific in the experiments conducted for this thesis suggests that a different, perhaps yet unknown co-factor(s) from those which Abid *et al.* (1) have considered, is responsible for this effect in plasma. It would be intriguing to determine if the accentuation effect associated with plasma

in this thesis is limited to only PrP^{Sc} seed from brain or would accentuate other PrP^{Sc} seed sources.

The plasma accentuation effect observed in this thesis most closely relates to a new prion conversion theory proposed by Abid *et al.* (2) in 2006, termed the 'nucleated-assisted' model. This model states that PrP^{C} binds to a conversion factor that results in an altered intermediate structural state of PrP^{C} thus allowing PrP^{C} to undergo the structural changes leading to PrP^{Sc} (2). This model differs from traditional models of conversion, the 'refolding' model, where PrP^{C} unfolds and refolds under the influence of PrP^{Sc} (73,185) and the 'seeding' or nucleation model which proposes that PrP can spontaneously assume different conformations (PrP^{C} and PrP^{Sc}) (53,73). It appears through this thesis work that a component(s) of plasma, an apparent species-specific conversion factor, is involved in the conformational change of PrP^{C} to PrP^{Sc}, but the involvement of plasma or its' component may be; a) direct interaction of misfolding PrP^{C} and propagation of PrP^{Sc} or b) indirect where a co-factor binding to an inhibitor of protein misfolding is disabled, allowing prion conversion to occur. In either instance, the data presented in this thesis demonstrates that plasma accentuates conversion, but the exact mechanism is unknown.

The accentuation effect observed with plasma was eventually limited, as a higher concentration (50%) of normal plasma added to the PMCA reaction had an inhibitory effect. If plasma concentrations were kept between 5 - 25%, >4.0 $\log_{10}$ PrP^{Sc} amplification after one round of PMCA (16 hours, 32 cycles) were observed. Contiguous plasma (*i.e.,* maintaining plasma in the NBH of subsequent

rounds of PMCA) led to even greater levels of PrP^{Sc} amplification reaching >6.75 log_{10} after two rounds of PMCA (32 hours, 64 cycles). A similar level of amplification had been reported by Castilla *et al.* (42) after 2 rounds, constituting 214 cycles of PMCA with only crude brain homogenate as the PrP^{C} substrate. Comparing the durations of PMCA (*i.e.*, 64 to 214 cycles), the plasma PMCA reported in this thesis was able to achieve a similar level of amplification as Castilla *et al.* (42) in a much shorter time (1.5-2 days rather than 4.5-5 days). The reduction in time required to produce similar levels of amplification with the plasma-specific PMCA could prove extremely beneficial when applied to time sensitive diagnostic tests.

To investigate if the plasma-specific PMCA could be optimized further, the duration of the PMCA rounds and presence of contiguous normal plasma for serial PMCA were re-evaluated. Increased PrP^{Sc} amplification was detected with extended duration (24 and 48 hours) of the 1^{o} PMCA, contrary to what had been observed during optimization of the basic protocol. The addition of a secondary round (additional 48 hours, 96 cycles) led to greater PrP^{Sc} amplification (>6.75 log_{10}) with contiguous plasma present in the NBH diluent of the subsequent round, but it was also observed that the NBH only control (*i.e.*, with no PrP^{Sc} seed) displayed a protease resistant band at the same molecular weight as PrP^{Sc}. The presence of contiguous plasma in sPMCA appeared to result in the *de novo* generation of PrP^{Sc}. When further independent experiments were done, the *de novo* PrP^{Sc} was detected as early as 2 rounds with 48 hours duration of PMCA (192 cycles) or 3 rounds of 16 hours of PMCA (96 cycles). This *de novo*

generation of PrP^{Sc} had also been observed by Deleault *et al.* (58) and Thorne and Terry (237). In the Thorne and Terry study (237), 7 $\log_{10}$ amplification of PrP^{Sc} was achieved after 2 rounds of PMCA (96 cycles) when synthetic poly A RNA was added to a blood leukocyte preparation that was used to seed the PMCA reaction (60). However after 3 rounds (144 cycles) of PMCA blood leukocyte fractions from sheep unexposed to prion disease displayed spontaneous conversion of PrP^{C}. Deleault *et al.* (58) also observed *de novo* PrP^{Sc} with non-denaturing PMCA after as few as 4 rounds (192 cycles). The appearance of *de novo* PrP^{Sc} in the negative controls in both studies was thus attributed to poly A and subsequently led to a novel approach of limited-sPMCA (40 cycles) to minimize the spontaneous conversion of PrP^{C} to PrP^{Sc} in plasma (199). Interestingly in the Deleault *et al.* (58) study of 10 independent experiments where *de novo* PrP^{Sc} was observed, *de novo* PrP^{Sc} appeared on average after 7-8 rounds of PMCA (as early as the 4^{th} round, but as late as the 12^{th} round). Based on the results presented in this thesis, the *de novo* synthesis of PrP^{Sc} found by Thorne and Terry (237) presumed to be associated with poly A in NBH preparations may be the linked to a component associated with blood and not isolated to the addition of poly A. It is possible that a similar co-factor associated with plasma (as has been shown in this thesis) contributes to *de novo* conversion of PrP^{C} and which may also be present in the blood leukocyte fraction of blood. The association of *de novo* synthesis with a co-factor that is associated only with blood products would explain the lack of *de novo* synthesis reported by Barria *et al.* (14) when poly A was present in standard PMCA reaction and a rationale for

the few rounds of PMCA required for *de novo* synthesis of PrP^{Sc} by Thorne and Terry in the presence of blood leukocytes (237).

As previously mentioned, in the Barria *et al.* (14) study, the addition of poly A to the PMCA reaction did not result in the spontaneous generation of PrP^{Sc}, but it was demonstrated that with further manipulation of the PMCA protocol *de novo* PrP^{Sc} could be produced. They determined that only after increasing the number of cycles from 144 cycles (standard PMCA) to 240 cycles (5 days) after 9 rounds of PMCA (*i.e.*, 45 days) was *de novo* generation of PrP^{Sc} observed in 20% of the hamster brain samples. The *de novo* generated PrP^{Sc} was inoculated into animals and caused disease in all cases. Unfortunately the study was unable to conclude whether the *de novo* product was the result of a PrP^{Sc} intermediate already present in the brain homogenate or if it was truly *spontaneous* misfolding induced by sPMCA. Other attempts to induce misfolding (high heat, addition of detergent, lower pH) in healthy brain homogenates failed to result in detectable *de novo* PrP^{Sc}. The findings presented in this thesis contradict those reported by Barria *et al.* (14), in that the simple addition of species-specific plasma component led to *de novo* synthesis of PrP^{Sc} and additional modifications to the basic PMCA protocol (increased heat, detergent, low pH) were not required. Replication of the generation of *de novo* PrP^{Sc} in a prion-free facility using the methodology outlined in this thesis eliminates the potential that this finding is the result of laboratory contamination (Appendix A.1)

Barria *et al.* (14) were also able to demonstrate that the *de novo* PrP^{Sc} product from their study was infectious and displayed several unique

characteristics implying it may be a different strain of prion disease. Increased Proteinase K (PK) sensitivity compared to other well established prion strains (including 263K) was one of the unique properties of the PMCA generated *de novo* PrP^{Sc}. Throughout this thesis work a higher concentration of PK (200 µg/mL), than is typically used (50 µg/mL), was maintained implying that the *de novo* PrP^{Sc} generated is highly resistant to PK. Determination of the PK resistance and infectious nature of the *de novo* PrP^{Sc} generated in this thesis may provide additional insight into variations in strain properties in a single species.

With detection of prion disease primarily limited to post-mortem detection in brain tissue, the next goal of this thesis was to perform experimental trials to determine the effectiveness of the plasma-specific PMCA with clinically-ill and control hamster plasma samples. In the two trials performed, PrP^{Sc} was detected in 82% of the samples tested after 6 rounds of sPMCA in the presence of contiguous plasma. Unfortunately, with the presence of contiguous normal plasma in the NBH diluent, all of the non-infected hamster plasma samples eventually led to *de novo* PrP^{Sc} synthesis as well. When the basic PMCA protocol (*i.e.,* without contiguous plasma present) was ran in parallel, no PrP^{Sc} was detectable from the infected or non-infected hamster plasma samples. The obvious disadvantage of the plasma-specific PMCA was that it could not distinguish infected from non-infected plasma samples before the appearance of *de novo* PrP^{Sc} formation. It cannot be determined if the *de novo* PrP^{Sc} generated stems from silent prions believed to lie dormant in the brain (261) and/or plasma, or if plasma truly induced the spontaneous conversion of PrP^{C} to PrP^{Sc}.

The complications with *de novo* synthesis of PrP^{Sc} with the plasma-specific PMCA make its' usefulness as a diagnostic tool for ultrasensitive detection of prions in plasma or other matrices limited. It exemplifies how caution must be exercised in the interpretation of results with *in vitro* conversion techniques, such as PMCA. With the application of a diagnostic test it is imperative to recognize and understand the potential complications (accentuation effect or *de novo* synthesis) that may occur with the test matrix (plasma or others). Application of novel ultrasensitive tools for detection of prion diseases is complex especially when crude brain homogenates are required as the platform substrate. The numerous interactions of the seed and substrate need to be considered along with the potential effects of various co-factor(s) interactions. In its' current state the plasma-specific PMCA cannot be directly applied to samples to distinguish between infected and non-infected individuals, but it has provided valuable information about the potential of a species-specific co-factor associated with plasma. The plasma-specific amplification accentuation was dependent on species-specificity of the plasma to substrate, this may partially explain the lack of published results with blood if the test sample (PrP^{Sc} seed) and substrate (PrP^{C} source) were not species, and potentially genotype (120,237), matched. Data from animal models or cell culture studying human prion disease must be interpreted cautiously as a lack of infectivity or PrP^{Sc} marker in association with plasma may be due to species barriers rather than a lack of disease.

Although the plasma-specific PMCA has its drawbacks it could be very beneficial if used with fewer PMCA rounds or limited-sPMCA, as in Rubenstein

et al. (199), and allow for detection with conventional validated diagnostic procedures (*i.e.*, WB), rather than a specialized detection system (SOFIA). Due to the increased sensitivity (>6.75 $\log_{10}$ after 32 hours [64 cycles] of PMCA) of the plasma-specific PMCA it may have more useful research applications. The plasma-specific PMCA may have considerable impact on the evaluation of technologies that claim to inactivate or reduce PrP^{Sc}, or where extremely low levels PrP^{Sc} are present. The plasma-specific PMCA would enhance the likelihood of detection with a minimal number of rounds of sPMCA and prove that the remaining PrP^{Sc} present may still have the ability to template disease. In addition the plasma-specific PMCA may provide an excellent system to study and/or manipulate the conditions of spontaneous disease.

Lastly the plasma-specific PMCA results illustrate the potential importance of plasma in disease progression and/or propagation. Understanding that PrP^{C} and PrP^{Sc} can cross the blood brain barrier (BBB) (8,9), it may be postulated that blood provides the necessary co-factors to trigger misfolding, as well as provide transportation of the misfolded product to the brain and systemically. Alternatively the PrP^{Sc} seed derived from the brain could cross the BBB accentuating misfolding in peripheral tissues in contact with blood (*i.e.*, spleen) during disease progression. Ingestion of PrP^{Sc} through dietary intake may lead to transport of the misfolded molecule systemically through the circulatory system, which in turn may accentuate the misfolding and spread of prion deposition in peripheral tissues. Moreover, this model of prion propagation may have several important implications in understanding other neurological protein

misfolding disorders. For example, brain trauma is a potential risk factor for amyotrophic lateral sclerosis (ALS) (46,56,75,96,181), and vascular leakage of plasma co-factors may facilitate misfolding or act as triggers of latent neurodegenerative disorders. Similarly, identification of this plasma co-factor(s) could serve as a therapeutic target for the potential treatment of prion/neurodegenerative diseases. The research presented in this thesis lays the foundation for further research in this area, and in a broader context towards application of the strategies used to adapt PMCA to detection of prions in other bodily fluids, tissues and/or complex matrices.

6 APPENDIX A

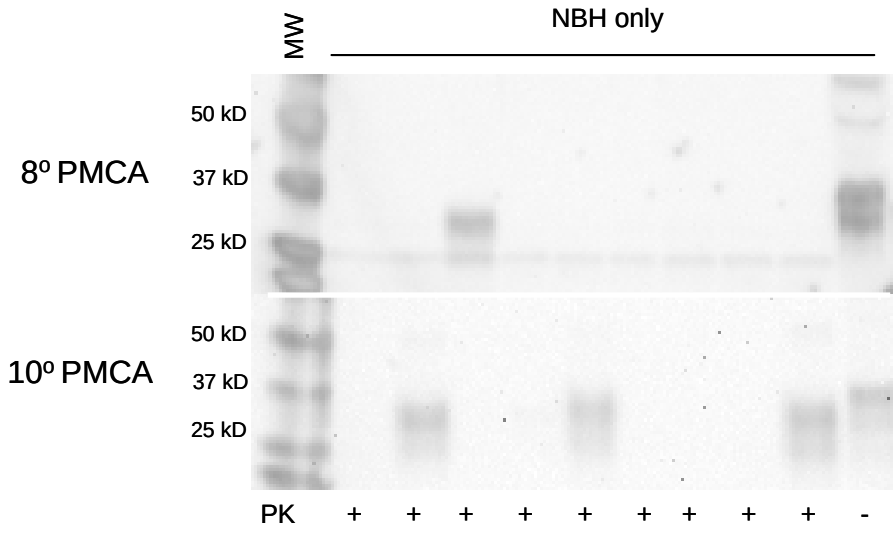

Figure A.1 Western blot demonstrating *de novo* synthesis of PrP^{Sc} in a prion free facility (Alberta Agriculture Level III, Edmonton, AB). Hamster normal brain homogenate (NBH) was prepared with 10% normal hamster plasma present (NBH only) and the 1^{o} PMCA performed, after each PMCA round (16 hours) the previous round PMCA product was diluted 1:10 into fresh NBH with 10% normal plasma present. Samples were processed with (+) and without (-) Proteinase K (PK) at 200 µg/mL. Lane labeled 'MW' represents molecular weight marker at 50, 37, 25 kD.

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