Assessing the risk to humans of transmission of novel TSE isolates by cell free conversion assays

Food Standards Agency Project M03055: Final technical report

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1. Executive Summary

Transmissible spongiform encephalopathies (TSEs) are fatal neurodegenerative diseases of humans, wild animals and domestic livestock that involve the conformational conversion of the prion protein, PrP^{C}, to a disease-associated form, PrP^{Sc}. PrP^{Sc} accumulates during the disease leading invariably to neuronal loss, neural vacuolation and death. The efficiency with which animal TSE diseases transmit to humans cannot be measured directly, hence model systems must be used to aid risk assessments, such as experimental infection of transgenic mice expressing human prion protein. In recent years, we have used an *in vitro* system, involving the conversion of recombinant prion protein by a seed of disease-associated PrP^{Sc}. The assay mimics many aspects of natural TSE disease, including the affect of prion protein polymorphisms and the inhibitory effect of various anti-TSE therapeutic compounds. Crucially, the cell free conversion assay (CFCA) system also appears to replicate species barriers. We have investigated the extent of protein conversion of human PrP in CFCA reactions seeded with PrP^{Sc} from novel, recently identified or experimental forms of TSE disease in animals. These diseases include atypical forms of scrapie and BSE. Traditionally, our CFCA uses radiolabelling as a method of detecting converted recombinant PrP against the background of PrP^{Sc}. However, because of the anticipated low conversion efficiencies, the need to prepare multiple different recombinant proteins for the assays and a desire to differentially detect two different proteins to mimic PRNP heterozygosity, we wished to develop fluorescent labelling techniques as a means of specifically detecting recombinant PrP. Despite much progress and encouraging results, we were unable to develop fluorescent labelling to a point where we believed that it could be used reliably in assays measuring cross species protein conversion. Relying on the traditional radiolabelling methodology, we radio-labelled 10 different recombinant PrP proteins and measured conversion efficiencies for human PrP proteins with 5 different sources of TSE disease. We find low conversion efficiencies which are, on the whole les than or equal to positive controls. Our data suggest that novel TSE isolates convert human PrP with no greater efficiency than cattle BSE suggesting that novel TSE isolates represent no more risk than BSE from cattle. These results should be interpreted side-by-side with *in vivo* studies in mice to determine overall risks of disease transmission.

2. Introduction

Transmissible spongiform encephalopathies (TSEs) are fatal neurodegenerative diseases caused by an aberrantly folded form of the prion protein, PrP^C. TSEs include scrapie in sheep and goats, bovine spongiform encephalopathy and various diseases in humans. The epidemic of bovine spongiform encephalopathy (BSE) in cattle in the 1980s and 1990s had a devastating effect on both the UK agricultural industry and society as a whole. Thousands of cattle were infected bringing the livelihood of many farmers into doubt and the subsequent epidemic of variant Creutzfeldt-Jakob disease (vCJD) in humans impacted on the national population, both through real and perceived risks of transmission. Although the vCJD epidemic has been mercifully small, there are still a small number of cases diagnosed each year and there is potential for secondary spread of disease from human to human through iatrogenic means, as evidenced by individuals apparently infected with vCJD after receiving blood transfusion[1]. Implementation of measures to prevent such a spread costs millions of pounds. The potential impact of any future TSE infection in humans is therefore high and the risk of TSE infection passing from animals, particular through consumption of meat products, needs to be as accurately assessed as soon as possible to allow suitable risk reduction measures to be implemented, as necessary. Thus, it is important to assess the risk to humans of any TSE identified in wild or domestic animals.

Recently, various novel TSE diseases have been detected in the UK, EU and worldwide[2]. These include atypical forms of both scrapie and BSE in sheep and cattle respectively. Classical scrapie has been endemic in the UK national flock for over two hundred years but epidemiological evidence suggests that this form of TSE disease cannot pass to humans. By contrast, BSE in cattle was demonstrably zoonotic, albeit with apparently low penetrance. It is not clear whether atypical scrapie[3] (also known as Nor98[4]) or atypical BSE (also known as BASE – bovine amyloidotic spongiform encephalopathy[5], which can occur as either L- or H-type) can pass to humans. Likewise, an epidemic of chronic wasting disease (CWD), a TSE of wild and captive cervids including deer, is currently sweeping through the US. This disease also has unknown zoonotic potential and, although it has not yet been detected in the EU, there remains risks that UK and EU cervids could become infected.

Unfortunately, there are few means by which one can accurately assess the potential for TSE diseases to pass between species. If the target species is animals then they can be inoculated with the exogenous TSE disease and the risk of disease transmission can be assessed, but such an experiment is clearly not possible when the species under test is

humans. *In vivo* alternatives involve the use of non-human primates as a model and some experiments have been done using squirrel monkeys to assess the efficacy of transmission of CWD infection[6]. These experiments are exceptionally expensive, take many years to complete and are ethically controversial. Other i*n vivo* alternatives involve the use of transgenic mice that have been modified to express human prion protein either from the endogenous promoter or from a cassette that results in over expression[7]. Since it is likely that other molecules are involved in the prion protein misfolded process, which will presumably still be 'murine-specific' in transgenic mice, the results of cross species transmission to humanised transgenic mice need to be interpreted with care. For example, certain results from use of humanised mice suggest that they are less susceptible to challenge with BSE/vCJD than their wildtype counterparts[8].

In vitro alternative systems also exist and techniques such as protein misfolding cyclic amplification (PMCA)[9-10] or quaking induced conversion (QuIC)[11-12] have been pioneered to mimic the misfolding process. Such techniques are not quantitative, however, making investigations of mechanism or of efficiency of cross species transmission difficult to achieve. We have traditionally used an *in vitro* model called the cell free conversion assay (CFCA), an assay that was pioneered during the 1990s by the group of Byron Caughey[13]. Briefly, the assay involves the conversion of recombinant PrP (recPrP) by a purified seed of PrP^{Sc} from a diseased animal[14]. This assay has been shown to qualitatively mimic many aspects of TSE disease, including the reduced conversion efficiency of recombinant protein carrying mutations known to confer resistance in animals and reduced conversion efficiency following cross species challenge. The assay is also inhibited by drugs shown to be effective anti-TSE agents[15-17] and conversion efficiencies can be measured with some accuracy. Thus, the CFCA provides a potential means to quantitatively assess the likelihood of transmission of TSE disease across species or to assess the role of prion protein point mutations occurring within a species on disease susceptibility. There exist some previous reports of cell free conversion assay variants applied to the study of cross species transmission of CWD[18-19] and previously the assay has been used to investigate the likely transmission of BSE and scrapie to humans[20].

2.1 The project - background

This project was established to use CFCA assays to provide assessments of the potential transmission of novel forms of TSE disease to humans. Other projects elsewhere were also established to use both gene-targeted and over expressing 'humanised' transgenic mice as *in vivo* models to access similar information. The CFCA experiments involve expression of

human recPrP; this protein is mixed with PrP^{Sc} purified from the brains of animals suffering from novel forms of TSE disease. In the CFCA, recombinant PrP is converted into a protease resistant isoform and the newly converted protein can be distinguished from the PrP^{Sc} seed by prior incorporation of a 'tag' into the recombinant PrP. Traditionally, the CFCA uses a radioactive tag – ^{35}S-methionine is incorporated into the recPrP during expression and this allows protease-resistant protein to be detected, after proteinase K (PK) treatment by SDS-PAGE and autoradiography. A schematic depicting the CFCA procedure is shown in figure 1. There are a number of problems associated with use of radioactivity as a means to detect recombinant protein. Radioactive substances are hazardous, incorporation into recombinant protein can be variable and detection by autoradiography is not always sufficiently sensitive, particularly for β-particle emitters such as ^{35}S. In addition, the half life of ^{35}S is measured in weeks and concomitantly preparing a large variety of radiolabelled proteins of sufficient activity is challenging.

For this project, we have only small amounts of infected brain material for some of the novel TSE disease isolates that we needed to test, which would challenge the sensitivity of autoradiography. We also needed to produce multiple different recombinant proteins at any one time to allow accurate comparison of conversion efficiencies within a single experiment. For these reasons, during much of the project period we have worked on development of an alternative method of tagging recPrP to allow detection after PK digestion involving cutting edge synthetic biology techniques and fluorescence-based labelling of proteins. Fluorescently labelled proteins have the distinct advantages that gel-based detection is often

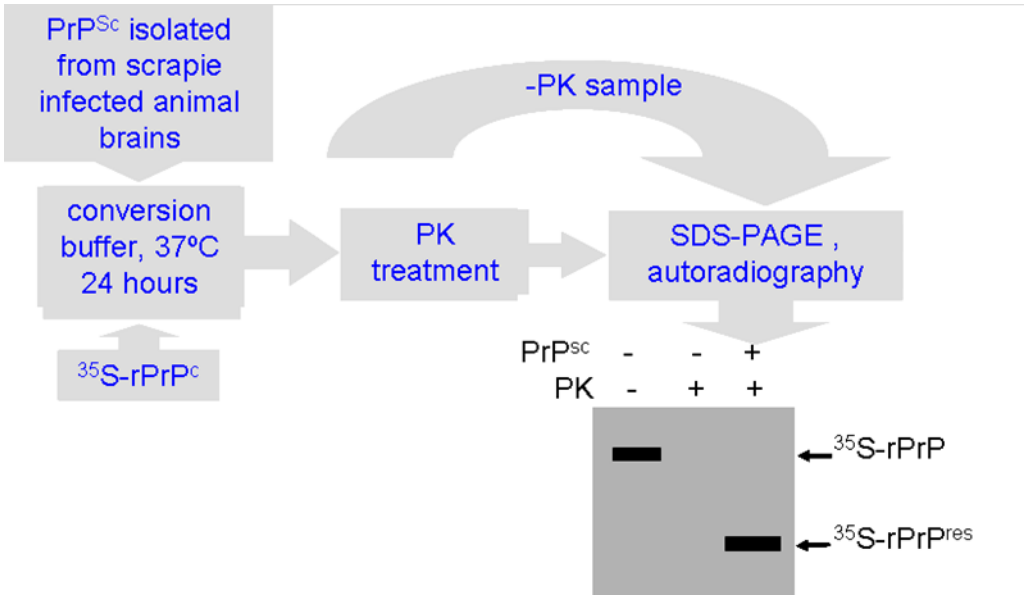


Figure 1 – Schematic representation of the traditional cell free conversion assay procedure

more sensitive than autoradiography and the fluorophores do not degrade over time in the same way as radioactivity is lost. They are also essentially non-hazardous.

Our strategy for fluorescently tagging recPrP was based upon amber stop codon suppression technology, using tRNA molecules engineered to be used by E.coli translation machinery but which recognises the TAG amber stop codon and incorporate an unnatural amino acid into the growing protein chain. The tRNA is reloaded with unnatural amino acid by an aminoacyl-tRNA synthetase that is expressed from the same plasmid as the tRNA[21]. We sourced the plasmids to allow us to do this from Peter Schultz (Scripps Institute, La Jolle). The novel TSE isolates to be used in this project were also sourced from other laboratories and represented those isolates that are of current interest to assess transmission characteristics to humans. They include atypical forms of scrapie and BSE. In the following sections we report results achieved during the course of the project and the estimates of conversion efficiency of human recombinant proteins by the novel TSE isolates. We find small but detectable levels of conversion associated with human PrP of the M129 genotype. Homozygous-FCA conversions show reduced conversion efficiency whilst conversions of V129 human PrP are close to zero. We find that novel isolates of TSE disease convert with no greater efficiency than cattle BSE and, in most cases, effiencies are reduced. Care should be taken when interpreting the results since *in vitro* systems are believed to model the effects of only the initial interactions between exogenous and endogenous protein and do not account for other *in vivo* effects, such as differential uptake of infectivity or rate of clearance of exogenous PrP^{Sc}. These data, therefore, should be used by comparison to data generated by other techniques, including transgenic mouse experiments.

2.2 Project Objective *– reproduced from original project submission*

The major scientific objective of this project is to provide estimates, based on quantitative measurements of protein structural conversion in vitro, of the likely susceptibility of humans to novel TSE isolates. These isolates include atypical scrapie, atypical BSE and CWD. The quantitative measurements will be compared against conversion of human protein by classical bovine BSE. We will also assess conversion efficiency of mixed protein preparations to mimic homozygous individuals (Met/Val codon 129) and also assess conversion efficiency of human protein carrying the P102L mutation.

3. Materials and Methods

3.1 Collation and analysis of infected tissue samples

Cattle brain tissue infected with primary BSE was sourced from archive materials, originally from the Veterinary Laboratories Agency (VLA). Ovine brain tissue infected with passaged BSE, from oral inoculation of sheep with BSE-infected cattle brain, was obtained from Nora Hunter, Roslin Institute. Ovine brain experimentally infected with atypical scrapie was obtained from Olivier Andreoletti (INRA-ENVT, France). Cattle brain infected with BASE was obtained from Cristina Casalone (CEA, Turin, Italy). Deer brain samples from white tailed deer experimentally infected with chronic wasting disease were obtained from Juergen Richt (USDA, Ames, Iowa; now KSU, US). We were unable to obtain sufficient quantities of vCJD-infected brain material to allow extraction of SAF.

3.2 Preparation of scrapie infected fibrils from infected tissue

Based on a method described by Hope *et al*.[22], SAF were isolated from the infected tissue samples collated as in section 3.1. A 5 % (w/v) homogenate was prepared from either 0.4 or 0.8 g of macerated brain material in a buffer containing 10 % (w/v) *N*-lauroylsarcosine, 0.1 M sodium phosphate, pH 7.4. The homogenate was centrifuged at 22,000 *g* for 30 min at 10 °C, the pellet was discarded and the supernatant centrifuged at 215,000 *g* for 150 minutes at 10 °C. The pellet was re-suspended in 3 ml H_2O followed by incubation at room temperature for 1 hour. To this, 9 ml of iodide solution (0.9 M potassium iodide, 15 mM sodium phosphate, 9 mM sodium thiosulphate, 1.5 % (w/v) *N*-lauroylsarcosine, pH 8.5) was added. The suspension was centrifuged at 285,000 *g* for 90 minutes at 10 °C through a cushion of 20 % (w/v) sucrose in 0.6 M potassium iodide, 10 mM sodium phosphate, 6 mM sodium thiosulphate, 1 % (w/v) *N*-lauroylsarcosine sodium salt, pH 8.5. The resultant pellet was washed in H_2O and centrifuged at 13,000 *g* for 30 minutes at room temperature. The final SAF pellet was re-suspended in 80 µl H_2O by sonication. Densitometric analysis of semi-quantitative Western blots incorporating serial dilutions of recombinant murine PrP gave an accurate concentration of PrP^{Sc} in each SAF preparation.

3.3 Expression and purification of recombinant PrP – general method

The expression, purification and refolding of recombinant prion proteins has already been described elsewhere[15]. For standard expression, bacteria were grown to an OD_{600} of 0.6-1.0 and protein expression was induced by the addition 1 M isopropyl-β-D-1-thiogalactopyranoside (IPTG) to a final concentration of 1 mM. Bacteria were cultured further overnight and harvested by centrifugation. The cell pellet was resuspended in 50 mM Tris-HCl, pH 8.0 containing 1 mM EDTA and 100 mM NaCl. Bacteria were lysed by the addition of lysozyme (final concentration 200 µg/ml) followed by addition of sodium deoxycholate (final concentration 1 mg/ml) and DNase (final concentration 10 µg/ml). Protein-containing inclusion bodies were harvested by centrifugation and were then solubilised in a buffer containing 8 M urea, 100 mM Na_2HPO_4, 10 mM Tris, pH 8.0. Recombinant protein was purified by binding to a column of immobilized nickel ions (Ni-IMAC, Qiagen), eluting bound proteins by step elution to pH 4.5. The protein was further purified by cation-exchange chromatography (SP

Sepharose, GE Healthcare), eluting bound components with a gradient from 0-500 mM NaCl. PrP-containing fractions were pooled, diluted to a concentration of approximately 0.1 mg/ml and 5-fold molar excess Cu^{2+} ions were added, overnight, to oxidize the disulphide bond. The final protein was dialysed extensively against 50 mM sodium acetate, pH 5.5 and was quantified by UV_{280} absorbance or by semi-quantitative Western blotting (as below)

3.4 Expression of site-specifically keto-tagged recombinant PrP

Plasmids expressing recPrP that incorporated an amber stop codon were prepared by site directed mutagenesis. We started from previously generated plasmids expressing wildtype PrP and following instructions outlined in the SDM kit (Qiagen). All mutated plasmids were checked by sequencing. For protein expression plasmids were cotransformed into DH10B expression bacteria with the pDule plasmids carrying tRNA and tRNA synthase genes[23] by electroporation.

DH10B expression bacteria were grown in 50 ml cultures of M63 minimal media containing ampicillin and tetracycline and 8 mM p-acetylphenylalanine (custom synthesised by Dr. Mark Thompson, Department of Chemistry, University of Sheffield) to an OD of 0.6-1.0. Expression was induced by addition of isopropyl-β-D-1-thiogalactopyranoside (IPTG) to a final concentration of 1 mM. Bacteria were cultured further overnight and harvested by centrifugation. Purification and refolding of recPrP followed that outlined above in section 3.3 except that home-packed 1 ml SPE tubes were used for both chromatographic steps.

3.5 Fluorescent labelling of keto-tagged recombinant PrP

RecPrP incorporating the unnatural amino acid p-acetylphenylalanine were fluorescently labelled in Starlab microcentrifuge tubes or deactivated glass vials (Waters). To approximately 200 µl recPrP (~0.1 mg/ml) were added Alexa fluor 488 hydrazine, Alexa fluor 555 hydrazine or Alexa fluor 647 hydrazine (Molecular Probes, Invitrogen) to a final concentration of 1 mM. The labelling reaction was incubated at either room temperature of 4 °C for 2 hours or overnight in the dark. Sodium cyanoborohydride was added to a final concentration of 5 mM and the reaction was incubated at room temperature or 4 °C for a further 1 hour. Excess reagent and reducing agent was removed by dialysis, desalting columns or HPLC as detailed in the results section. Fluorescently labelled protein was stored at 4 °C prior to use.

3.6 Cell free conversion assays

Details of our cell free conversion assay methodology have previously been published[14]. Briefly, recombinant proteins were produced as above (section 3.3), except bacteria were grown in M63 minimal media supplemented with S^{35}-methionine during induction of protein expression. This yielded radiolabelled recombinant protein, which was mixed with a seed of fibrils purified from the brains of terminally TSE-infected mice, by a procedure involving detergent extraction and differential centrifugation (section 3.1). The mixture of proteins was incubated at 37 °C for 24 hrs and was subsequently treated with proteinase K (PK), after prior removal of an aliquot equal to 1/10th of the

reaction mixture for use as a –PK control. All samples were separated by SDS-PAGE and gels were imaged and analysed according to one of the following procedures.

3.7 Detection of fluorescently labelled protein by fluorescence scanning

Fluorescently tagged protein was analysed by SDS-PAGE followed by fluorescence scanning by use of either a Biorad MultiImager FX scanner or a GE Healthcare Typhoon scanner. Excitation was performed by use of a laser of appropriate wavelength (488, 532, 647 nm) and emission filters appropriate for the fluorophore being detected were used.

3.8 Detection of radiolabelled protein by autoradiography

After separation of conversion assay products by SDS-PAGE, radiolabelled recPrP was detected by autoradiography using a phosphor screen (GE Healthcare). Typically, CFCA gels were exposed to the phosphor screen for 3 days to allow the signal to develop and the phosphor screen was then imaged using either a Biorad MultiImager FX scanner or a GE Healthcare Typhoon scanner. Excitation was done by use of a laser of wavelength 647 nm and proprietary emission filters suitable for phosphorimaging screens were used.

3.9 Densitometry and analysis

Protein bands on scans of Western blots and phosphor screens were quantified by densitometric methods using ImageQuant software (GE Healthcare). For quantitation of both PrP^{Sc} isolated in SAF preparations and starting radiolabelled recPrP, standard curves were produced corresponding to serial dilutions of wildtype recPrP. For CFCA quantitation, for each pair of samples we set the densitometric intensity of the control sample (-PK) to 100% and calculated the percentage of protein left after PK treatment based on densitometric intensity.

4. Results

As outlined in the introduction, this project involved cell free conversion assays to investigate likely cross species transmission of novel TSE isolates to humans. The resources required for this project were sources of each novel TSE isolate, recombinant prion proteins from multiple species including humans and an assay capable of delivering a quantitative estimate of conversion efficiency of the human recPrP with each isolate. To provide a context, based on real transmission efficiencies that can be estimated, the conversion of human proteins by cattle BSE was used. The following sections outline the steps that were undertaken to achieve the final outcomes making reference to the objectives in the original project plan.

4.1 Relevant infected brain samples were compiled

Typically, cell free conversion assays are seeded with scrapie associated fibrils isolated from the brains of individuals infected with TSE disease. For each individual CFCA assessment, 1 µg of PrP^{Sc} is required; based on previously determined yields of 10 µg per mouse brain (which weigh roughly 500 mg) we determined that we would need in excess of 2-3 g of infected brain material for each isolate to be tested. Even then, depending on yield of fibrils, this quantity would not necessarily ensure success.

We sourced brains of animals that were infected with CWD, atypical scrapie and L-type BASE and also sourced tissue from sheep infected with BSE. As a control we sourced brain tissue from cattle infected with primary BSE from the time of the BSE epidemic. This agent has been demonstrated to have passed to humans, thereby causing the vCJD epidemic. However, it is assumed that transmission levels are relatively low since much of the UK population was presumably exposed but there have been few primary vCJD cases (at least to date).

The following table outlines the isolates that we received:

Disease isolate	From	Type of disease	Quantity
Cattle BSE	VLA archive material	Natural disease	~35 g
Sheep BSE	Nora Hunter, The Roslin Institute	Experimental infection of ARQ/ARQ sheep	~ 5g
Atypical scrapie	Olivier Andreoletti, INRA-ENVT France	Experimental infection of AHQ/AHQ sheep	15 g total from two brain regions

Disease isolate	From	Type of disease	Quantity
BASE	Cristina Cassalone, CEA, Turin, Italy	Natural case	2 g
CWD	Juergen Richt, USDA, Ames, Iowa, USA	Experimental infection of white tailed deer	~3 g

All tissues were stored at -80 °C until use. Unfortunately, we were not able to obtain vCJD brain samples in sufficient quantity to allow extraction of fibrils suitable for conversion assays.

4.2 SAF could be prepared from all infected TSE isolates

Using our traditional CFCA protocols, we prepare fibrillar seeds from mouse brain pools of 4-8 at a time, i.e. a starting wet tissue weight of 2-4 g. This was not possible for the tissue isolates used during this project as a result of the low starting weight of materials and restrictions on the available resources in our containment level 3 laboratories. Hence we used a scaled-down version of the SAF preparation procedure which started with wet tissue weights of 0.4 or 0.8 g and prepared multiple batches of SAF. In all cases, we prepared tissue macerates of significantly greater amounts of tissue than this to minimise batch-to-batch variations in the yield of fibrils. Apart from this difference, and variations in the rotors and tubes used for ultracentrifugation steps, we performed standard protocols to prepare SAF from each of the infected tissue isolates that we sourced. All work was done at containment level 3. In many cases this is, to the best of our knowledge, the first time that SAF have been prepared from such novel TSE isolates for biophysical/biochemical studies. Nevertheless, SAF preparation on all 5 TSE isolates resulted in a visible final pellet, which was always resuspended in 80 µl of water. From this, 2 µl were withdrawn and were subjected to SDS-PAGE and Western blotting using an anti-PrP monoclonal antibody alongside serial dilutions of recPrP standards. Following imaging and densitometric analysis, this process allowed semi-quantitative estimates of the amount of PrP^{Sc} present in each of the preparations.

In order to ensure sufficient PrP^{Sc} was available for the CFCA we performed repeat preparations of SAF from various tissue isolates and each time performed semi-quantitative Western blotting. Typical results from this procedure are shown in figure 2 (a-e) for each of the disease isolates used. Additionally, we confirmed that the signals from SAF were PrP^{Sc} by PK treating the final SAF preparation pellet and performing Western blotting to detect PK-resistant PrP^{Sc}, as shown in figure 3 for ovine BSE and CWD.

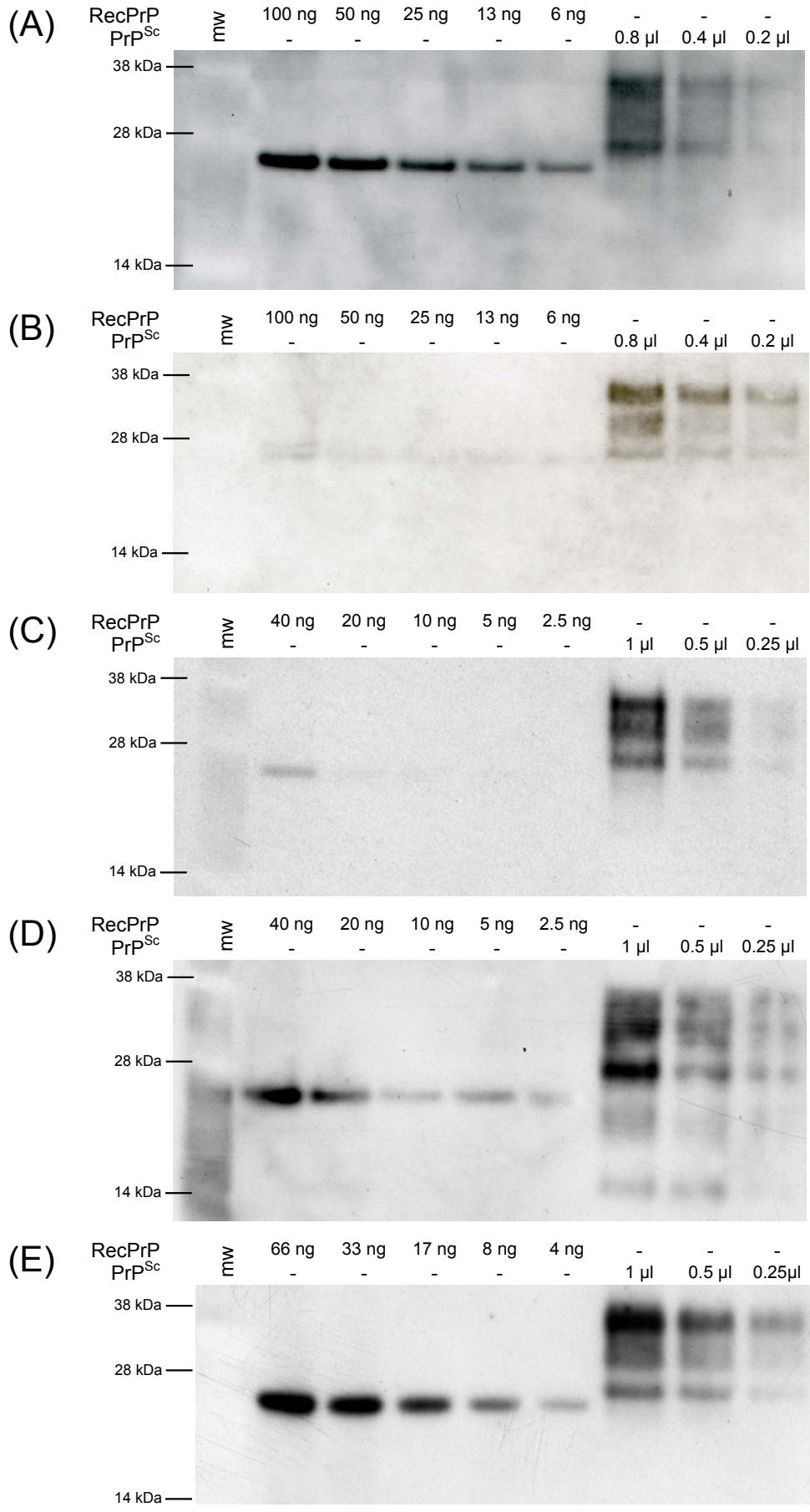


Figure 2 – Semi-quantitative Western blots of recPrP dilution series alongside SAF isolated from (A) cattle BSE (B) ovine BSE (C) CWD (D) BASE (E) atypical scrapie. In each case the amount of recPrP in each lane is indicated and the quantity of SAF, in µl, out of a 80ul suspension at the end of the SAF preparation protocol.

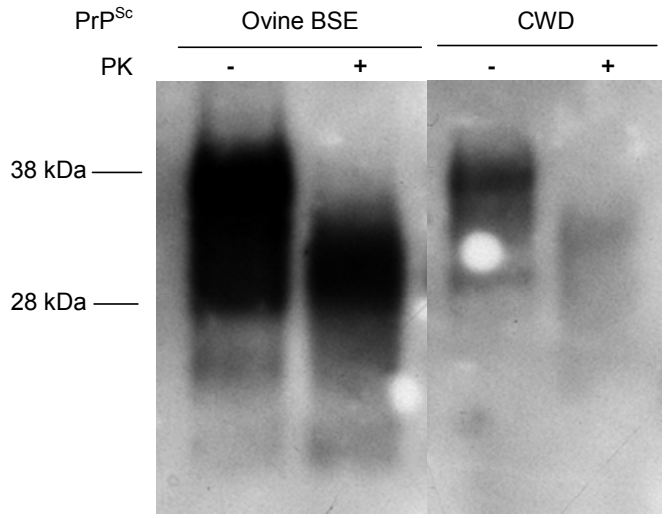


Figure 3 – Composite Western blot of SAF preparations from ovine BSE and CWD both with and without treatment with proteinase K.

These results demonstrated that each of the infected tissue isolates could be used to prepare SAF; by using repeat preparations we achieved the levels of SAF required for subsequent SAF work. These data demonstrate that we achieved objective 01/01.

4.3 Plasmids expressing human RecPrP were constructed and expression optimised

In order to undertake all conversion assay work detailed in the project outline, we needed to produce a range of different recombinant proteins. Aside from human recPrP (the common variants Met129, Val129 and variants containing the disease-associated mutation M129/P102L, V129/P102L) we also needed to produce proteins that would form positive controls for our conversion assay work. We required ovine protein of the ARQ genotype as a control for ovine BSE CFCA and ovine protein of AHQ and ARQ/L141F genotypes as control for ovine atypical scrapie assays. We needed cattle protein for controls for both cattle BSE and BASE conversion assays and finally needed deer protein for positive control CFCA reactions seeded with CWD. Since the effect of some commonly-occurring polymorphisms on the susceptibility of deer to CWD is still the matter of some debate[24], we decided to express two different deer proteins to increase the chances of getting a positive conversion reaction seeded with CWD fibrils.

In previous work, we had already cloned the wildtype cattle protein (BovPrP) and ovine proteins of genotype ARQ (OvPrP_ARQ) and AHQ (OvPrP_AHQ). During this project period we additionally created bacterial constructs expressing ovine PrP with the L141F polymorphism (OvPrP_ARQ_L141F), associated with susceptibility to atypical scrapie and the wildtype white-tailed deer protein (CePrP) and Q213E variant (CePrP_Q220E). In all

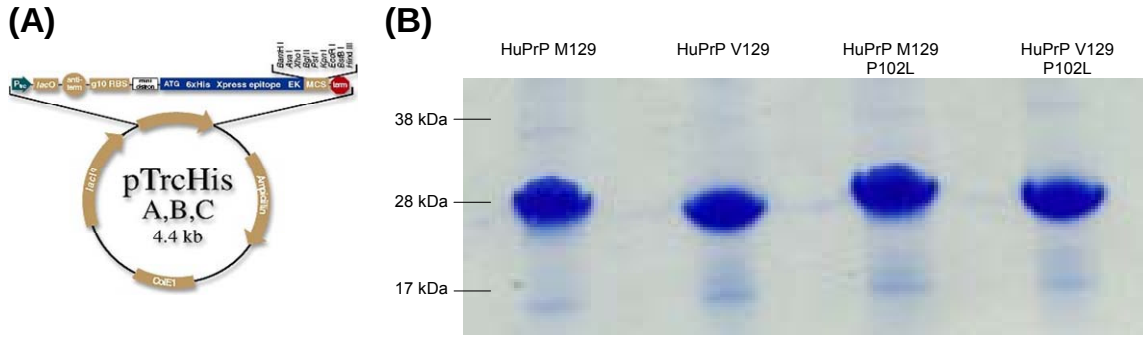


Figure 4 – Expression of human recombinant proteins (A) The open reading frame encoding the mature length human PrP protein was inserted into the multi-cloning site of the pTrc vector after prior removal of the hexa-histidine tag. Appropriate modifications were made using site directed mutatgenesis to allow expression of the human PrP variants M129, V129, M129/P102L and V129/P102L (B) After transformation of Rosetta expression bacteria with pTrc plasmids encoding the 4 human PrP variants, protein expression was induced and recPrP was purified from the inclusion body pellet using procedures detailed in the materials and methods section. The coomasie-stained SDS-PAGE gel shows the final products of this procedure to demonstrate excellent purity and good expression levels.

cases, the open reading frame for mature length PrP, i.e. without the N- and C-terminal signal peptides, was cloned into the pTrc vector after removal of the hexa-histidine tag. This produced proteins that commenced with an initiator methionine residue but then encompassed the entire mature PrP protein (figure 4(a)). Full sequences of proteins used in this project are detailed in Appendix A.

Our initial attempts to express the 4 human proteins (HuPrP_M129, HuPrP_V129, HuPrP_M129_P102L, HuPrP_V129_P102L) required in this project were only marginally successful. We used our standard expression bacteria, 1B392, but yields of human proteins were poor. Additionally, we continually observed two isoforms of the protein, as determined by SDS-PAGE, after purification. During this project we optimised the expression of human proteins – a prerequisite to produce sufficient protein during the less efficient labelling steps – by trialling different expression bacteria. We found that Rosetta bacteria (Invitrogen) were optimal for expression of all human recPrP proteins and achieved excellent expression levels and purity, as demonstrated in figure 4(b). For all proteins we ensured correct sequence by sequencing DNA and correct protein folding by CD. For the majority of the proteins we ensured correct expression by mass spectrometric analysis of refolded products.

4.4 Expression of recombinant prion protein incorporating a site-specific ketone group was successful

After successfully sourcing infected tissue samples, demonstrating high-yield, reproducible SAF preparation and producing the bacterial expression constructs required during the project, we sought to develop methods to fluorescently label the recPrP that we

would use in conversion assays. As outlined previously, we considered this to be an important element of the project to allow us to generate all 10 protein variants sequentially without concerns over the loss of radioactivity from any one protein. Fluorescence detection would also allow greater sensitivity than is typically possible using autoradiographic applications. Various potential strategies exist for fluorescent labelling of proteins, including the use of succinamide derivatives for targeting free amines, such as those on lysine side chains, or reagents that target free thiol groups. However, such reagents tend to act on all such functional groups in a protein and the result is a poorly structured protein and one which would almost definitely not be amenable to conversion seeded with PrP^{Sc}.

Our strategy for fluorescently labelling recPrP involved the site-specific incorporation of a functional group that is not present in normal proteins – the ketone functionality. Since ketones are not present on any normal amino acid side chains, if one could be engineered into recPrP then it would be unique within the protein and reagents that target ketones and aldehydes – hydrazines, hydrazides and hydroxylamines – would react only in the location chosen by us. Technology to allow site-specific incorporation of a keto-containing amino acid in bacteria has previously been developed by the group of Prof Peter Schultz (Scripps Institute, La Jolle) and we obtained reagents from him for use in our laboratory[21, 23]. The technology is based upon amber stop codon suppression by use of tRNA molecules that are orthogonal to normal *E.coli* translation machinery. The tRNA molecule is accompanied by a matched aminoacyl-tRNA synthetase (RS) that is also orthogonal to *E.coli* machinery and which has been selected to deliver a particular unnatural amino acid to the orthogonal tRNA. Thus, we obtained from Schultz's lab a plasmid that, when transformed into expression bacteria:

1) will express tRNA and RS that are not recognised or used by normal E.coli translation machinery
2) will produce tRNA that is loaded with the unnatural amino acid p-acetyl phenylalanine (pAPA) – see figure 5(a)
3) will use the orthogonal tRNA to incorporate pAPA into proteins in response to an amber stop codon

A basic schematic outlining this approach is shown in figure 5(b)

We identified various sites within PrP that could potentially be used to attach a fluorophore. These sites were chosen based on the arguments that the pAPA would (i) be replacing another large, hydrophobic amino acid (ii) would be oriented with the side chain partially solvent exposed and (iii) would be in the C-terminal domain that is retained after PK

digestion of protease-resistant protein. We selected the sites 198 and 231 (murine numbering) and performed site-directed mutagenesis to create plasmids in which the wildtype codon was replaced with a TAG amber stop codon. We also obtained synthetic pAPA from collaborators in the department of Chemistry, University of Sheffield. For all initial development work we focussed on use of murine proteins, since the CFCA systems using recombinant murine PrP seeded with either 87V or ME7 strains of mouse passaged scrapie are particularly well characterised and typically result in efficient conversion.

After co-transformation of bacteria with both the PrP expression plasmid and the pDule plasmid expressing the orthogonal tRNA and RS genes, we performed various trial expressions of the two proteins variants. We determined that both expressed satisfactorily and selected the variant with pAPA incorporated at codon 198 (mouse numbering) to take forward. This codon is within the 2nd N-linked glycosylation consensus sequence and we argued that linking a fluorophore in this region may simulate the glycosylation normally seen in mammalian expressed PrP thereby representing a benign location at which to anchor a fluorophore; substitutions in this region should affect neither structure nor ability to convert, as evidenced by high levels of glycosylation in PrP^{Sc} isolated from brain. We performed experiments to optimise expression of this protein and to optimise the incorporation of the pAPA unnatural amino acid during expression.

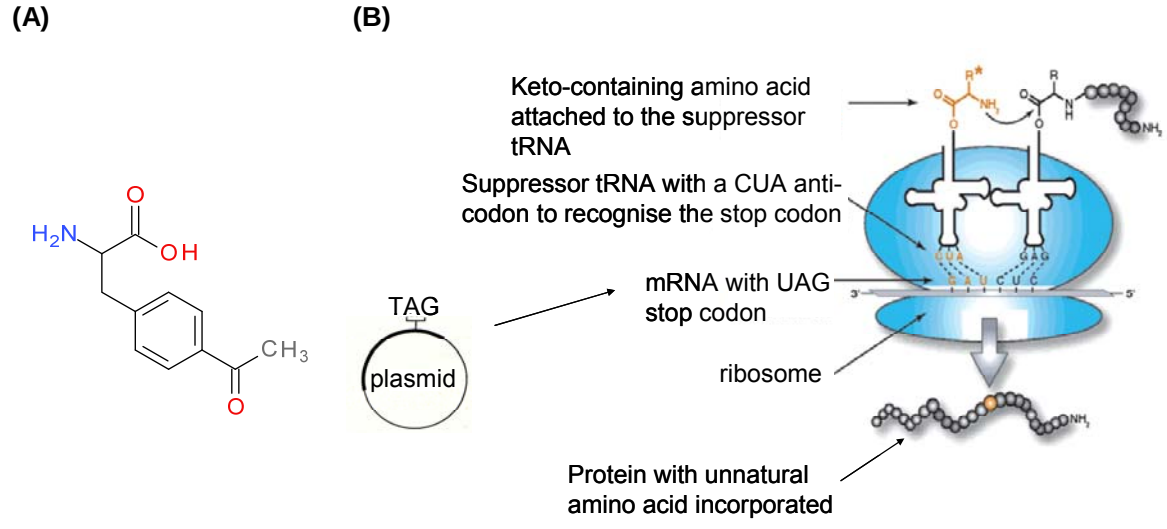


Figure 5 – (A) Chemical structure of p-acetyl phenylalanine, the ketone –containing unnatural amino acid to be incorporated into recPrP. (B) schematic outlining the concepts involved in site-specific incorporation of unnatural amino acids into proteins in response to an amber stop codon. tRNA that recognises the UAG stop codon in mRNA is loaded with the unnatural amino acid by a specific tRNA synthetase. During protein translation, the tRNA delivers the unnatural amino acid to the growing peptide chain specifically in response to the amber stop codon.

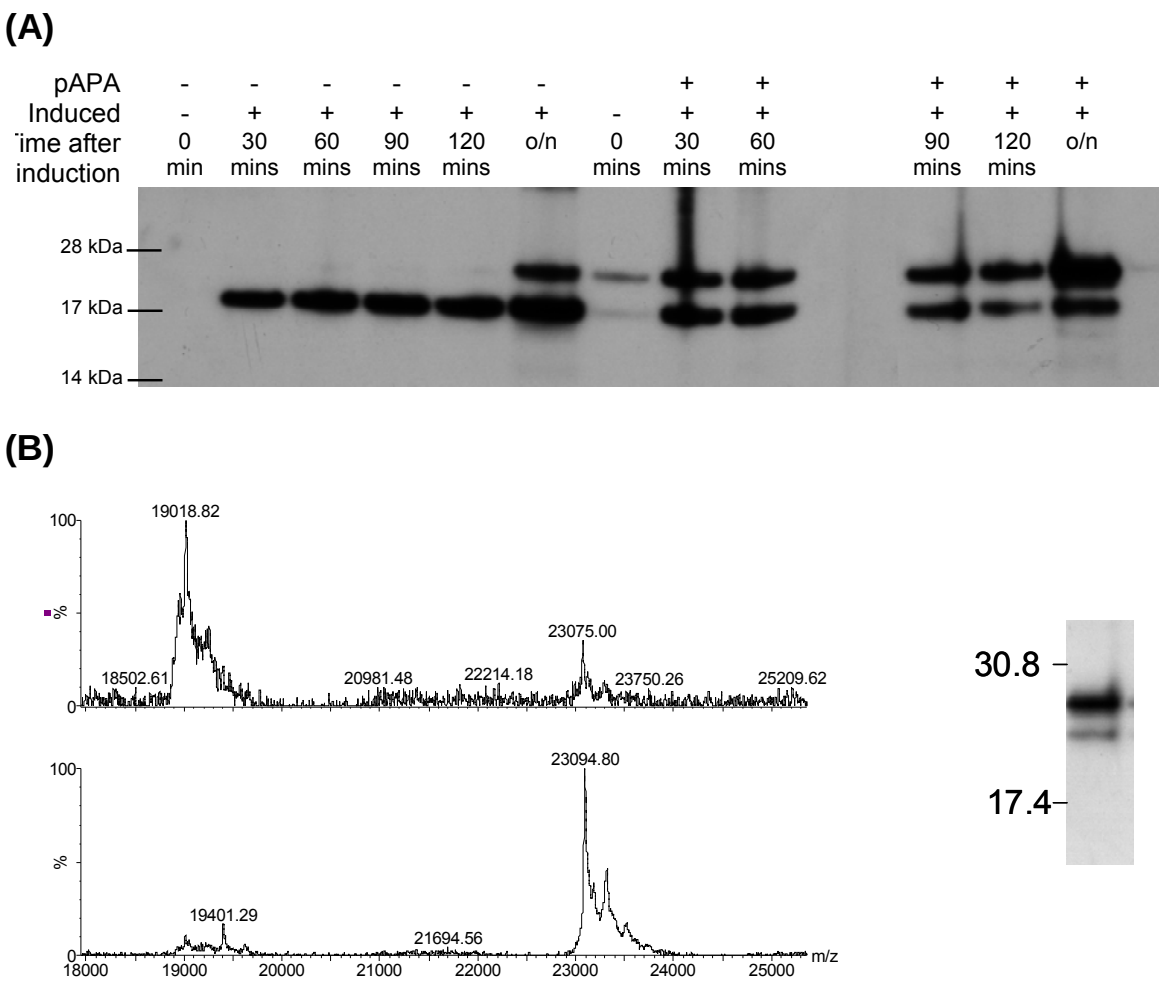


Figure 6 – (A) Western blot of trial-sized expressions of murine recombinant PrP incorporating the unnatural amino acid p-acetyl phenyl alanine (pAPA) at codon 197. In the absence of induction of expression little recPrP is detected. After induction in the absence of pAPA a single low molecular weight band is evident corresponding to protein in which expression has stopped at residue196. A small amount of full length recPrP is evident after overnight (o/n) induction. In the presence of pAPA, the higher molecular weight isoform predominates, particularly after overnight induction. (B) mass spectra of 198TAG protein expressed in the absence of the unnatural amino acid (top) and in the presence of the unnatural amino acid (bottom) in the bacterial culture medium. Without the amino acid expression stops at codon 198 whereas when unnatural amino acid is supplied expression yields ~85% full length protein. The accompanying SDS-PAGE gel provides an additional measure of relative yields of the two protein forms in the presence of unnatural amino acid

An example of the results of such an experiment, as determined by Western blotting, is shown in figure 6(a) and illustrates the specificity of this approach. Without induction of protein expression, extremely low levels of protein are produced (figure 6(a), lanes 1 & 7). After induction, but in the absence of pAPA in the culture medium, a protein is produced of approximate molecular mass 20 kDa. This protein corresponds to PrP residues 23-197 at which point expression stops in response to the amber stop codon. Taking samples at

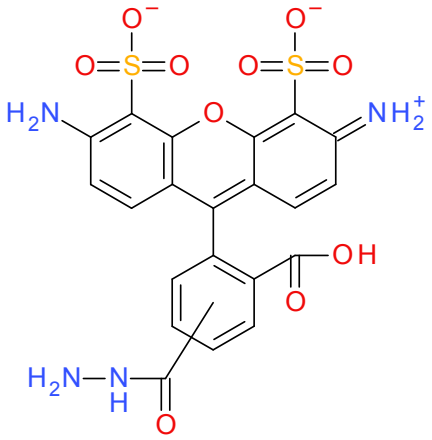
various times after induction reveals that this protein form builds gradually, although after overnight induction a small amount of a higher molecular mass form is also present. This corresponds to full length PrP into which, presumably, a normal phenylalanine has been incorporated in response to the stop codon. In contrast, in the presence of pAPA, the higher mass form is produced soon after induction and, after overnight induction, reasonable quantities of this form are produced.

The expression protocol was further optimised to increase yields of the high molecular mass isoform and we analysed the product by mass spectrometry to check that pAPA was indeed being incorporated into the protein. Figure 6(b) shows the mass spectrum of the protein and the measured mass of 23096 Da is in reasonable agreement of the predicted mass (23103 Da) and is significantly higher than the measured mass of either wildtype protein or protein expressed in the absence of pAPA. These data suggest the correct expression and incorporation of pAPA was achieved. We also demonstrated correct expression of the protein variant which incorporates pAPA at the C-terminus. Thus, this technology appears to be an excellent way to create a PrP molecule to which fluorophores can be linked by hydrazine chemistry.

4.5 Linking of fluorophores to ketone-containing recombinant PrP was optimised and proceeded in reasonable yield

The chemical reaction between functionalised amine derivatives and aldehydes/ketones is exceptionally useful, since it is highly specific and proceeds at a range of pHs. Many of the Alexa fluor series of dyes are available as hydrazine, hydrazide or hydroxylamine derivates and can be used directly for coupling to keto-containing substances, such as our site-specifically tagged recPrP. Figure 7(a) shows the chemical structure of Alexa Fluor 488 hydrazine and 7(b) outlines the chemical reaction that occurs between hydrazine and ketone compounds. For reactions of this type, the exact solution conditions require optimising based on the chemistry and physical/biophysical properties of the substance to be labelled, in this case recPrP. Thus, we performed a variety of different experiments to investigate the optimal pH, time, fluorophore concentration and temperature for the fluorophore labelling step. In each case we monitored the formation of fluorescently tagged recPrP by use of SDS-PAGE monitored by fluorescent scanning but in many cases also used the same gel for silver staining. This allowed us to compare fluorescence and non-fluorescence based detection of the same proteins to give partial quantitation of the yield of fluorescently tagged protein relative to levels of unlabelled protein.

(A)



(B)

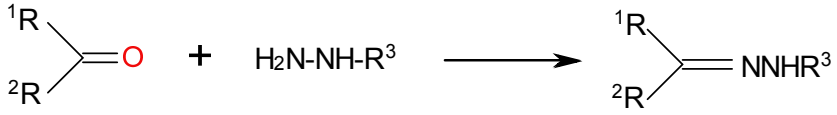


Figure 7 – (A) Chemical structure of Alexa Fluor 488 hydrazine. (B) The chemical reaction that occurs between ketone and hydrazine derivatives

For initial experiments we started with Alexa fluor 488 with which to label the protein. Examples of the SDS-PAGE results of labelling optimisation experiments are shown in figure 8. This data is from experiments in which we tested the efficiency of fluorophore labelling at different pHs, for differing lengths of time and with different quantities of fluorophore (molar excess over the recPrP substrate). We determined that labelling was most efficient at ~pH 5.5 and that labelling reactions should be left at least overnight for optimal labelling. We determined that there was no major advantage to using greater than ~50 fold molar excess fluorophore, at least in terms of yield of fluorescent product. We also used different fluorophores – Alexa fluor 555 and Alexa fluor 647 – to label the recombinant protein and demonstrated that these fluors also gave reasonable levels of labelling. Crucially, these reagents produced proteins that were detected by use of different scanning modes allowing differential detection of labelled proteins, as shown in figure 9 for protein labelled with 488 and 555 fluors. This was believed to be important, but not essential, to allow additional insights to be gained from conversions employing mixtures of proteins that would mimic PRNP heterozygosity.

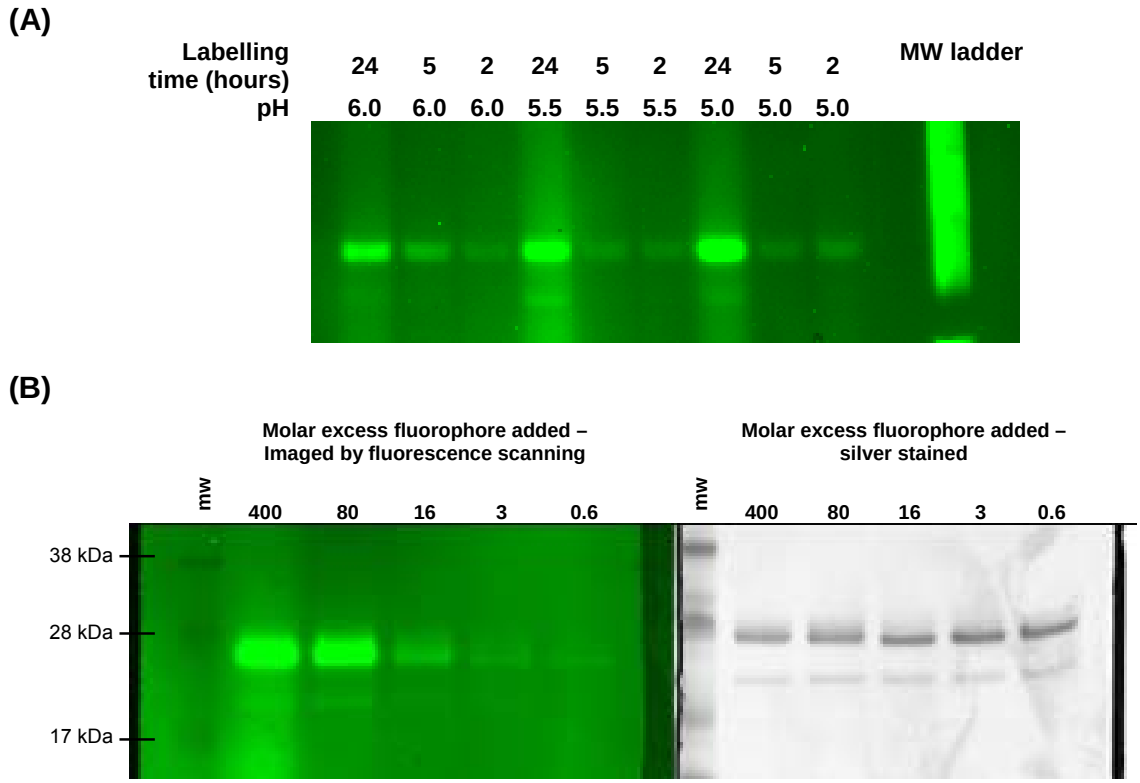


Figure 8 – (A) Variation of the efficiency of fluorescence labelling as a function of incubation time and pH. Three reactions were set up at different pHs and samples of equivalent volume were withdrawn for analysis at the times stated. The SDS-PAGE gel was imaged by fluorescence scanning for Alexa fluor 488 (B) Variation of the efficiency of fluorescence labelling with different recPrP:Alexa fluor 488 stoichometries. After labelling, a single SDS-PAGE gel was performed with replicate samples. The gel was dissected longitudinally and one half was imaged by fluorescence scanning for Alex Fluor 488, whilst the other was silver stained to detect total protein.

During these experiments, however, we encountered major problems with loss of protein during and after fluorophore labelling, often characterised by reduced or zero signals on SDS-PAGE gels after labelling. We noticed that this was frequently accompanied by discoloration of the Eppendorf tubes in which the reaction took place and, less frequently, by a visible precipitate in the tubes. These problems hampered reproducibility of analysis. To attempt to maintain the solubility of the fluorescently labelled product, we trialled labelling of recPrP at reduced temperatures. Incubation at 4 °C gave less precipitate but overall yields were reduced and still rather variable. This was most marked when preparing dilutions of fluorescent PrP to determine detection limits – signals dropped exponentially, which can be explained more by loss of protein than by the anticipated reduction of detection associated with serial dilutions, which would give a more linear response.

To further attempt to improve yields and stability of our labelled recPrP proteins, we asked whether the plastic composition of Eppendorfs and pipette tips adversely affects the fluorescently labelled proteins. We prepared serial dilutions of Alexa fluor labelled recPrP in

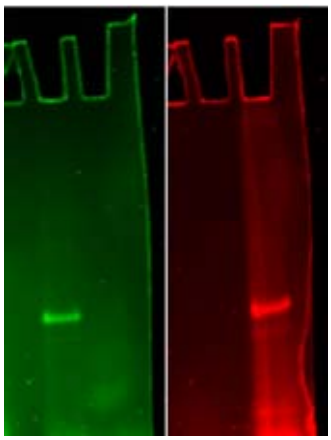


Figure 9 – labelling of recPrP with two different fluors. recPrP was labelled with either Alexa fluor 488 or Alexa fluor 555 and both proteins were analysed by SDS-PAGE. The gel was imaged using fluorescence scanning under conditions to detect either the 488 fluor (left image) or the 555 fluor (right image). This demonstrates the specificity of detection and that both fluors can be used to label recPrP.

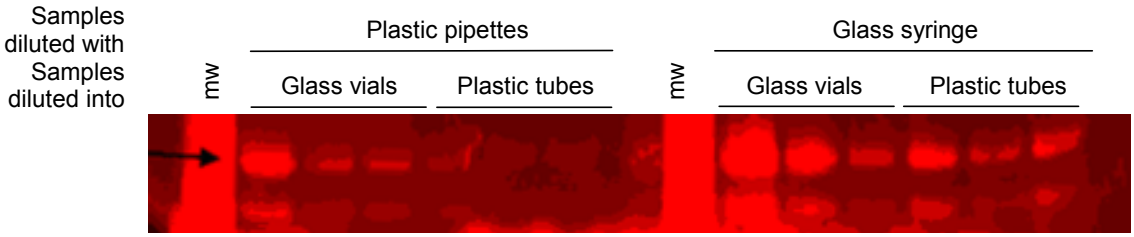


Figure 10 – SDS-PAGE gel of an experiment to test whether Alexa fluor 555-labelled recPrP adheres to plastic. Serial dilutions of labelled recPrP were made into either plastic Eppendorf tubes or glass vials. For dilutions, samples were transferred with either plastic pipette tips or a glass hypodermic syringe. The results demonstrate that more protein is retained for gel analysis after dilution by use of hypodermic syringes into glass vials.

either plastic Eppendorf tubes or glass vials; to transfer liquids either plastic pipette tips or glass hypodermic syringes were used. The results, shown in figure 10, suggest that the protein is more stable and adheres less to glass vessels than to plastic vessels, including pipette tips. After these experiments we used only glass vessels or specially pre-treated centrifuge tubes for incubations and glass hypodermic syringes for all transfers of liquids containing fluorescently labelled proteins. We also incorporated several other steps within the overall process of labelling and analysis of fluorescent protein: the double bond formed between protein and fluorophore (Figure 7(b)) can be stabilised by reduction and we incorporated a treatment with sodium cyanoborohydride to reduce this bond; we also reduced the labelling time slightly to try to reduce the levels of precipitation/adsorption to tubes that was occurring during long reactions. The final protocol gives labelling that proceeds in reasonable yield and the fluorescently labelled protein is detectable to low levels, as shown in figure 11, which represents serial dilutions of ovine protein labelled with either Alexa fluor 555 or 647 at the C-terminus (codon 234).

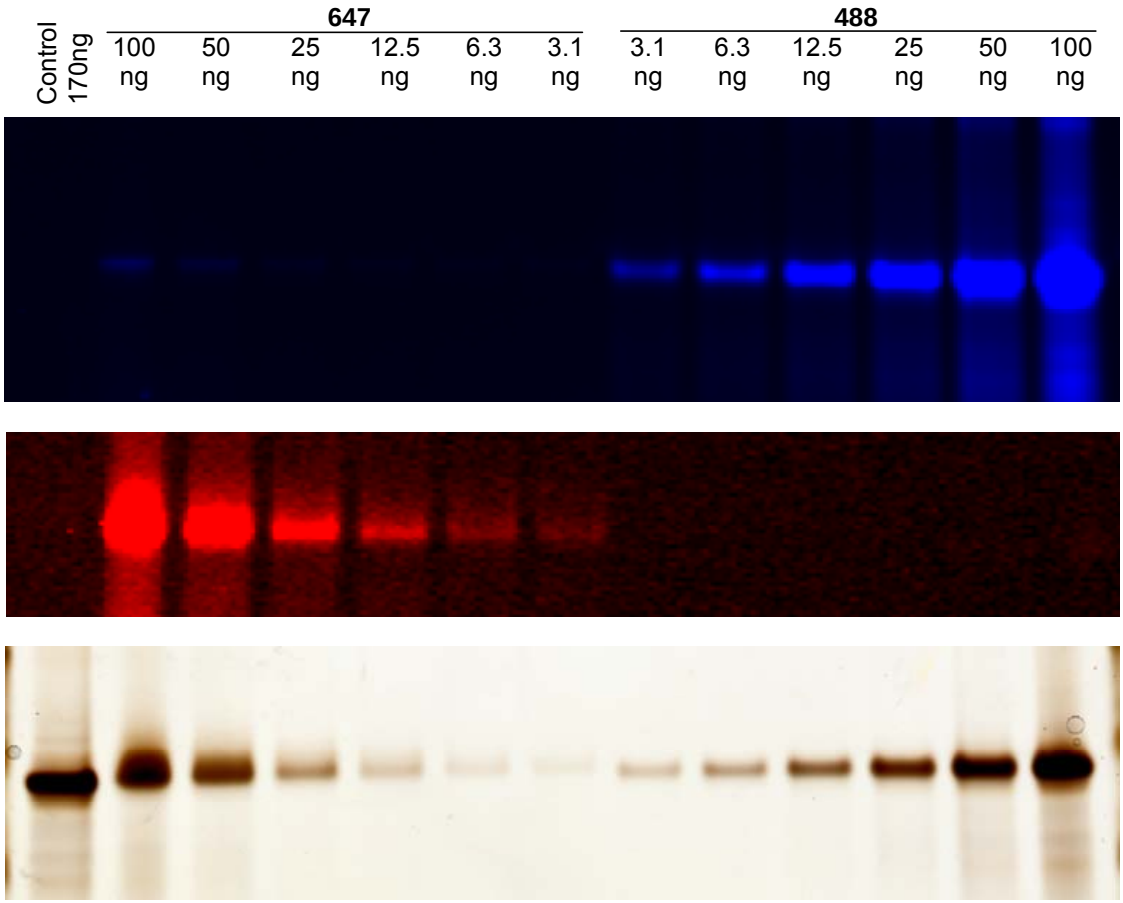


Figure 11 – dilution series of OvPrP234TAG labelled with Alexa fluor 488 or Alexa fluor 647 and images using settings to detect 488 fluor (top) 647 fluor (middle) or silver stained (bottom). Protein concentrations were measured by densitometry of silver staining intensity compared to unlabelled recombinant protein controls in a separate experiment.

Next, we preceded to trial cell free conversion assays by use of the fluorescently labelled proteins seeded with mouse-passage scrapie. Initial results were promising and showed the presence of bands within the fluorescently imaged SDS-PAGE gels at approximately the correct molecular weight. Results are shown in figure 12 that indicate that cell free conversion in the presence of PrP^{Sc} appears to give a protease-resistant product and that the products are detectable only by imaging under the correct conditions for each of the two fluorophores. These are indicated with arrows on the gels shown in Figure 12. When a mixture of proteins composed of equal amounts of recPrP labelled with 488 or 555 fluorophores is used then bands are detected under both scanning conditions. To our knowledge, this is the first time that fluorescent-based detection has been used in conversion assay applications. However, the gel images presented in figure 12 are characterised by excessive background and fluorescence from other proteinaceous species and more optimisation needed to be done before these proteins could be used for real CFCA experiments. Nevertheless, these results demonstrate the feasibility of this approach and fulfil objective 02/01 and partially fulfils objective 02/02.

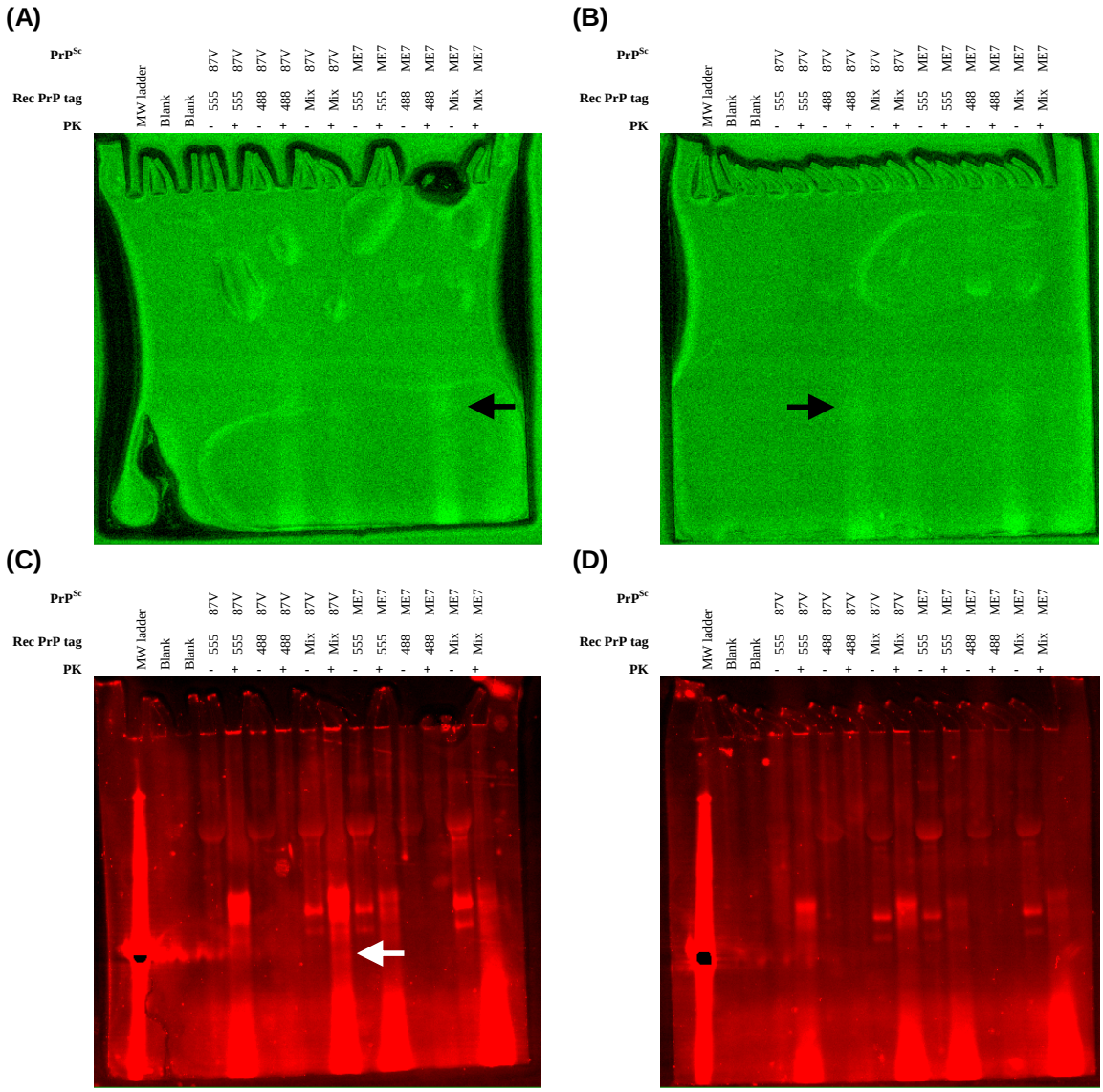


Figure 12 - Cell free conversion assays of fluorescently tagged recombinant protein substrate, converted with seeds of either 87V or ME7 fibrils. Cell free assays were done with proteins labelled either with Alexa Fluor 488, Alexa fluor 555 or with mixtures of the two proteins. Gels were detected either under conditions to detect 488-labelled protein (A) and (B) or 555-labelled protein (C) and (D). Gels in panels (B) and (D) and replicates of gels in panels (A) and (C). Arrow heads point to specific bands that represent fluorescently labelled, PK resistant protein. Crucially, we get full discrimination between 488 and 555 labelled protein, even in mixtures. These data demonstrate that cell free conversion using fluorescently labelled proteins is feasible and that it could be used, with two different protein preparations, to mimic heterozygosity.

4.6 Cell free conversion assay of fluorescently tagged protein was problematic despite optimisation of solution conditions

The most likely reason for extraneous signals on initial CFCA gels is that there is free, unreacted fluorophore still present in the CFCA solution that has carried over from the labelling step. Hence various techniques were trialled to attempt to remove free fluorophore from the labelling reaction prior to setting up the CFCA reactions. The direct approach of dialysis to remove free reagent was attempted, but this approach resulted in significant loss

of protein, presumably through binding to the dialysis tubing, which had visible signs of discolouration after use. We also attempted PD10 desalting columns as per the manufacturer's instructions. However, we found a similar loss of protein during the desalting step and overall yields were extremely poor as determined by SDS-PAGE and both fluorescence scanning and silver staining. Both techniques were trialled repetitively under different conditions with no gains of final yield.

Encouraged by results of other purification protocols ongoing in the laboratory, we tested an approach based upon reversed phase HPLC purification; the fluorescence labelling reaction mix was loaded onto a C_4 HPLC column, in the presence of 0.01% trifluoroacetic acid, and bound components were eluted with a gradient of increasing acetonitrile. Fractions were collected across the course of the elution and analysed by SDS-PAGE and fluorescence imaging/silver staining. Typical results are shown in figure 13. Panel A shows the UV trace of the HPLC elution. The large peak at ~6 minutes corresponds to free dye, whilst later eluting peaks correspond to proteinaceous species. Fluorescent scanning of an SDS-PAGE gel (panel B) demonstrates conclusively the presence of fluorescently labelled protein in fractions 12-16, whilst the free dye was predominately present in fraction 2, as evidenced by the strong signal at the bottom of the gel. Fractions containing labelled PrP were lyophilised to remove organic solvent and acid and this procedure resulted in a coloured film of protein indicative of the presence of fluorescently labelled recPrP.

Purified, lyophilised protein was resuspended in water and used for additional trial cell free conversion assay trials. A typical result is shown in figure 14 and these data show that we have successfully removed free reactive fluorophore from the recPrP substrate, as evidenced by much cleaner gels with less background and less additional protein signals. However, disappointingly we achieve essentially zero conversion of the fluorescently labelled protein in this system and the only PK-resistant band is also present in control CFCA reactions that lack the PrP^{Sc} seed. We note that the recPrP substrate has been lyophilised and resuspended in water as part of the purification protocol. It is possible that this process interferes with the normal, α-helical conformation of recPrP and we tested this by repeating the HPLC separation and lyophilisation and then analysing the resuspended product by circular dichroism spectropolarimetry. The results (shown in figure 15) indicate that the resuspended protein adopts a predominately β-sheet structure, as evidenced by a single minimum in the spectrum at ~215 nm. α-helical proteins produce CD spectra characterised by a double minimum at ~222 nm and 208 nm. Thus, it appears that the HPLC purified and lyophilised protein is a poor substrate for CFCA as it lacks the normal helical

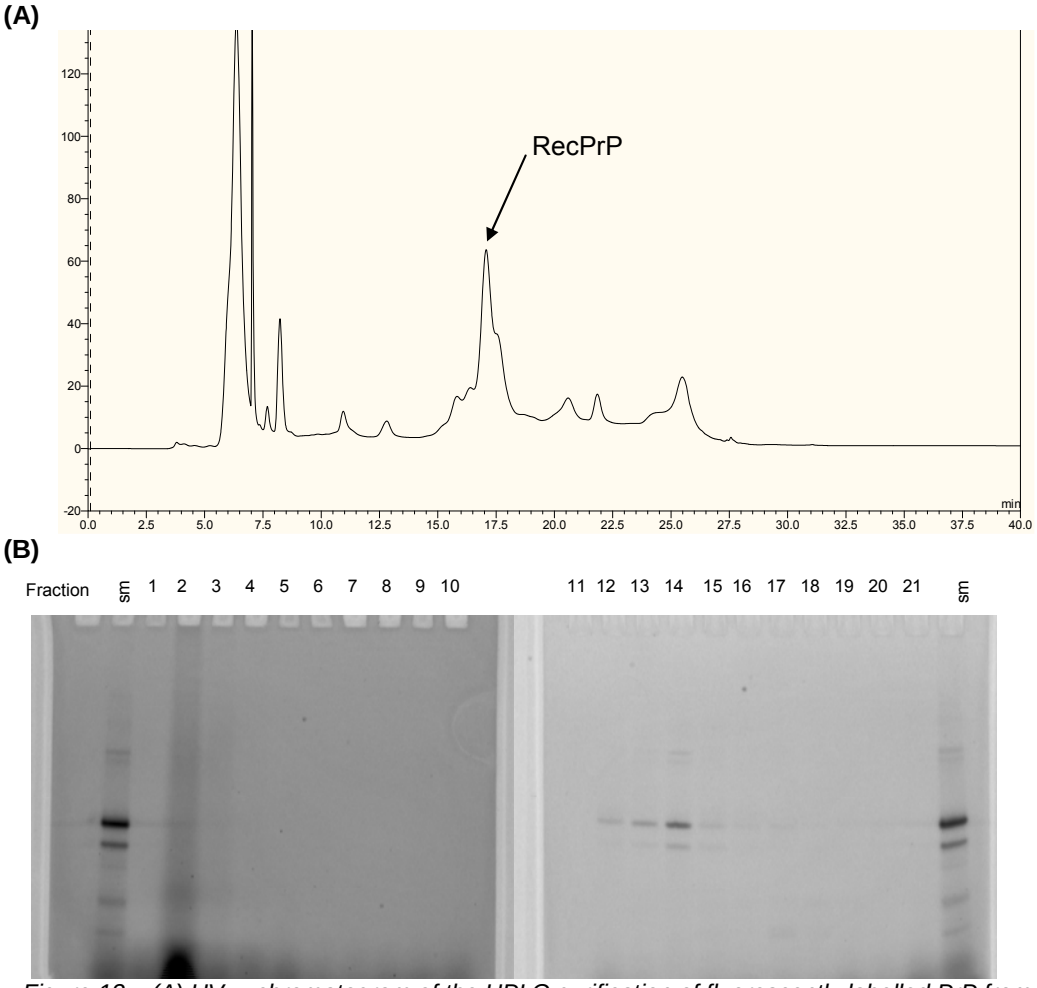


Figure 13 – (A) UV_{280} chromatogram of the HPLC purification of fluorescently labelled PrP from free fluorophore. Fractions of 1 ml volume (equivalent to 1 min elution time) were collected starting from 5 minutes. (B) Fluorescently scanned SDS-PAGE image of gels of HPLC fractions.sm= starting material. Purified fluorescently labelled PrP is predominately in fraction 14, corresponding to the HPLC peak at ~17.5 mins

fold. We have recently demonstrated the importance of a correctly folded and stable substrate for efficient conversion in the CFCA[17, 25] and the current data reaffirm this.

If the HPLC purification methodology produces a protein that is not correctly structured, what other procedures could be adopted for removal of free reactive fluorophore? We had already trialled dialysis, desalting columns and HPLC-based purifications and there are few other procedures that could be used on protein that adheres to plasticware and that we only have microgram quantities of. Ongoing work is dealing with this issue more fully. Nevertheless, in the current project period, we finally tested a range of other reagents that react with ketone derivatives to determine whether these would have more utility in CFCA to specifically detect converted recPrP. These included the use of fluorophores with different length linkers, to investigate whether the proximity of the fluorescent group to the protein was causing the conformational change. We achieved excellent labelling efficiency with some of these reagents (figure 16) but CFCA with one of these was also unsuccessful. We

also tried to couple a biotin moiety to the ketone derivative, which also proceeded successfully, but detection of the resulting reagent, through streptavidin conjugated to HRP, was insufficiently sensitive for use and, besides, this technology would also not allow differential detection of different protein variants. At this stage in the current project we abandoned attempts to use fluorescent tagging of recPrP as a means of detection in favour of the traditional approach of radiolabelling even though we recognised that production of all recombinant proteins needed, with suitable levels of radioactivity to permit low level detection, was especially challenging.

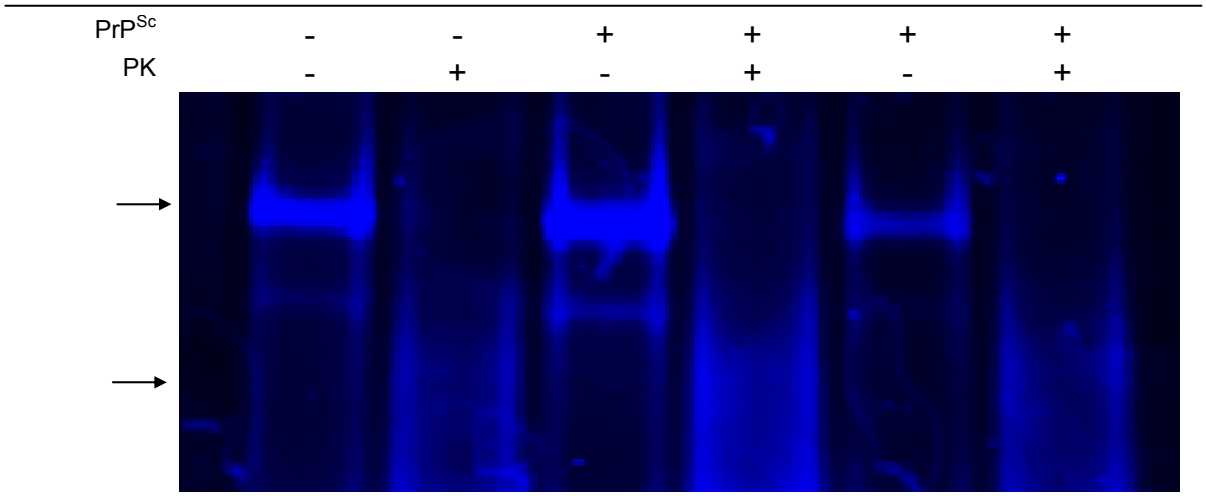


Figure 14 – cell free conversion assay of murine recPrP with Alexa fluor 488 at codon 198 seeded with 87V scrapie. Starting recPrP is clearly detectable (top arrow) but signals corresponding to PK-resistant products (bottom arrow) in lanes 4 and 6 are not significantly different from the negative control reaction (lane 2).

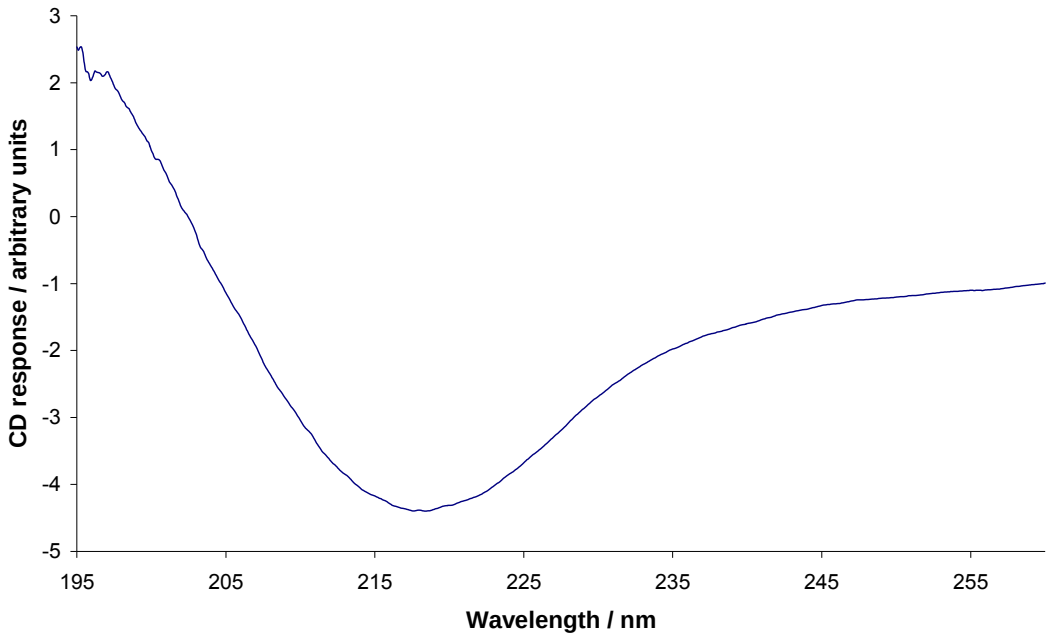


Figure 15 – Circular dichroism spectrum of Alexa fluor labelled recPrP. The single minimum in the region 215-220 nm is indicative of beta-sheet conformations

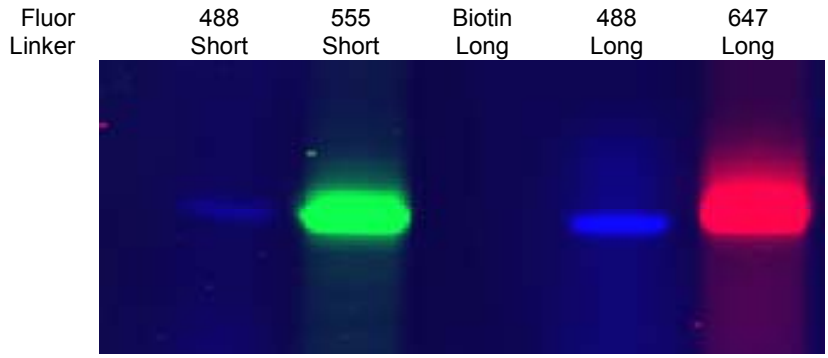


Figure 16 – Labelling and subsequent detection of ovPrP234TAG with various different reagents demonstrating specificity of approach.

4.7 Multiplexed expression of radiolabelled recombinant prion protein produced reagents for cell free conversion assays

Including all protein variants for positive control reactions, there were a total of ten recombinant proteins needed for the cell free conversion assays in this project, as detailed above in section 4.3. Typically, radiolabelling is carried out with 18.5 MBq of ^{35}S-methionine per protein preparation. Due to restrictions over the amount of radioactivity that can be stored, handled and disposed of in any one calendar month, we split the production of these ten proteins into three batches or four, two and four protein variants respectively. This also necessitated the use of multiplexing when expressing and purifying recPrPs. All work was carried out in the radioactivity laboratories at The Roslin Institute but supplies of equipment in these laboratories are limited and there are clearly tight controls over where and how radioactivity can be used. As a result, we have no gel images of the purification of the proteins, which were carried simultaneously for all proteins in a batch using gravity flow columns of 1 ml volume. The low column volumes allowed greater concentrations to be achieved than usually results from such a process. We monitored the eluate of both nickel ion affinity and cation exchange chromatography columns by Geiger and, in some cases, scintillation counting, to allow the correct recPrP containing fractions to be identified and

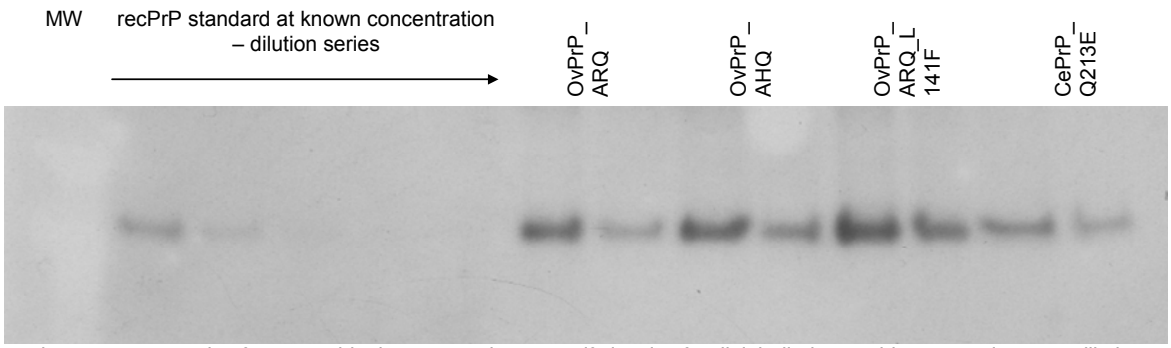


Figure 17 – Example of Western blotting approach to quantify levels of radiolabelled recombinant proteins. Two dilutions of various recPrP variants were loaded onto an SDS-PAGE gel alongside dilutions of a kown concentration of recPrP standard. Semi-quantitative Western blotting allowed estimates to be made of radiolabelled recPrP concentrations

pooled those fractions with high counts for the next stages. After refolding and dialysis of the proteins, we analysed the final products by SDS-PAGE and Western blotting to enable an estimate of final protein concentration to be derived by comparison to dilutions of non-radiolabelled recPrP standards. This mirrored our standard method for quantitation of PrP^{Sc} present in fibril preparations and an example of this approach for some radiolabelled recPrP variants is shown in figure 17.

In general, protein concentrations were ~50 ng/µl or greater and emission activities were of the order of 200-400 cps/ng. However, yields of the two human proteins carrying the P102L mutations were consistently lower than of unmodified proteins and activity levels were not sufficient to give clear detection after phosphorimaging applications. Regrettably, we were therefore unable to carry these proteins through to CFCA analysis.

The batches of proteins prepared were as follows:

Batch 1: HuPrP_M129; HuPrP_V129; HuPrP_M129-P102L; HuPrP_V129_P102L
Batch 2: BovPrP; CePrP
Batch 3: OvPrP_ARQ; OvPrP_AHQ; OvPrP_ARQ_L141F; CePrP_Q220E

It should be born in mind that the halflife of ^{35}S is such that proteins expressed in batch 1 would have considerably reduced activity by the time of CFCA analysis than those expressed in later batches. Nevertheless, apart from the P102L variants, all other proteins expressed well, gave good yields and activity was sufficient to allow analysis.

4.8 Cell free conversions with a subset of recombinant proteins revealed the extent of conversion with PrP^{Sc} seed from different species

Using radiolabelled recombinant protein as substrates and purified SAF from novel TSE isolates as seed, we set up a range of cell free conversion assays, including appropriate controls for both positive and negative conversion reactions. We set these up in replicates to allow us to generate two different conversion efficiencies per assay. Following published protocols (see section 3.6), after the assay we withdrew an aliquot of the reaction mixture equal to $1/10^{th}$ of the volume and treated the remaining $9/10^{th}$ with proteinase K. Samples were precipitated by treatment with ethanol and resolved by SDS-PAGE. Radiolabelled proteins were detected by phosphorimaging – the screen was scanned using a Biorad MultiImager FX, as detailed in section 3.8.

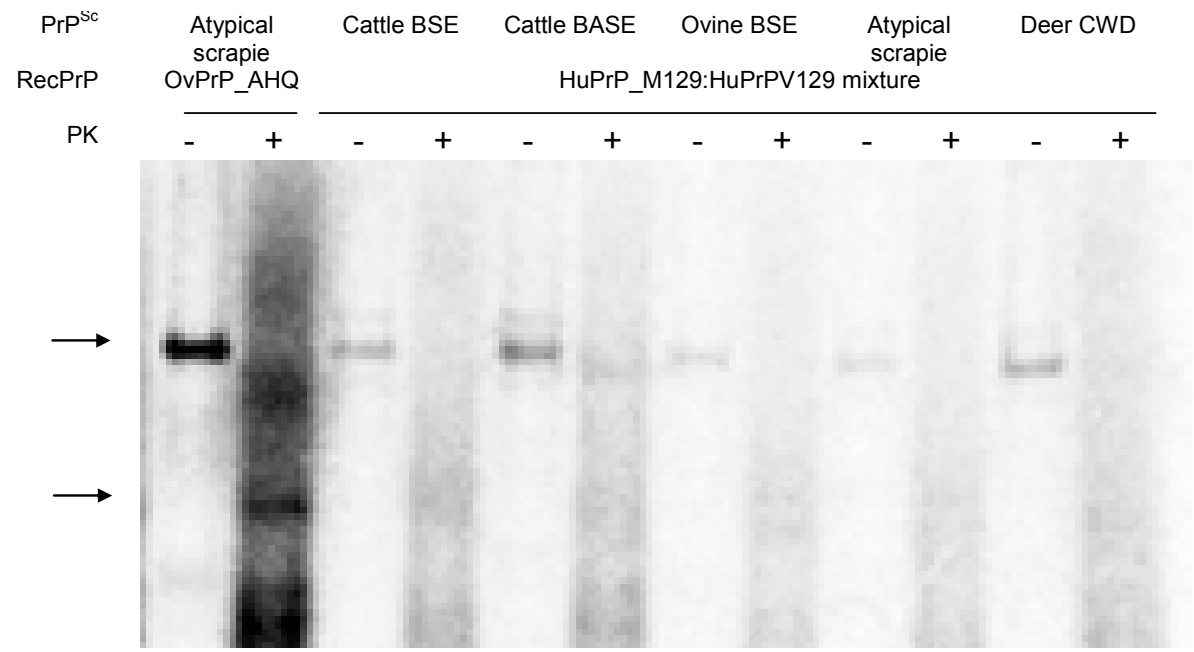


Figure 18 – Example cell free conversion assays of a 1:1 mixture of M129 and V129 human PrP seeded with all 5 TSE isolates along with a positive control reaction of OvPrP_AHQ seeded with atypical scrapie. Radiolabelled recPrP substrate is detectable (top arrow) whilst faint bands corresponding to PK-resistant recPrP are also detectable after PK treatment (bottom arrow)

Figures 18 shows selected images from cell free conversion assays. These images were used for densitometry using the ImageQuant software. As a result of low overall conversion efficiency, as would be expected for cross species CFCA experiments, it was difficult to conclusively identify which band in the phosphorimaging related to the PK-resistant converted product. Based on previous experience we would expect this to resolve at ~17.5 kDa. In the majority of positive control samples, a band is evident around similar to 17.5 kDa and we have used this band to generate the estimates of conversion efficiency that we are most confident about. However, a higher molecular mass band is also present in the majority of lanes and we have also used this band to generate conversion efficiencies that we believe are less likely. However, we also report these since we believe that it is important not to remove data that may be real. Using the densitometry software package ImageQuant, we measure pixel intensities for all putative bands in all lanes. An example of this approach is shown graphically in figure 19 for one of our positive control reactions. From this data, we were able to subtract data from negative control lanes to generate normalised, background subtracted conversion efficiencies for a variety of combinations of seed and substrate. The following table represents the mean conversion efficiencies determined in our experiments (NT indicates efficiencies that have not been tested. The first number represents conversion efficiencies that are associated with the lower band, whilst the number in brackets represents conversion efficiency associated with the higher PK resistant band). Note that levels of BASE fibrils were insufficient to allow all combinations to be tested, hence we

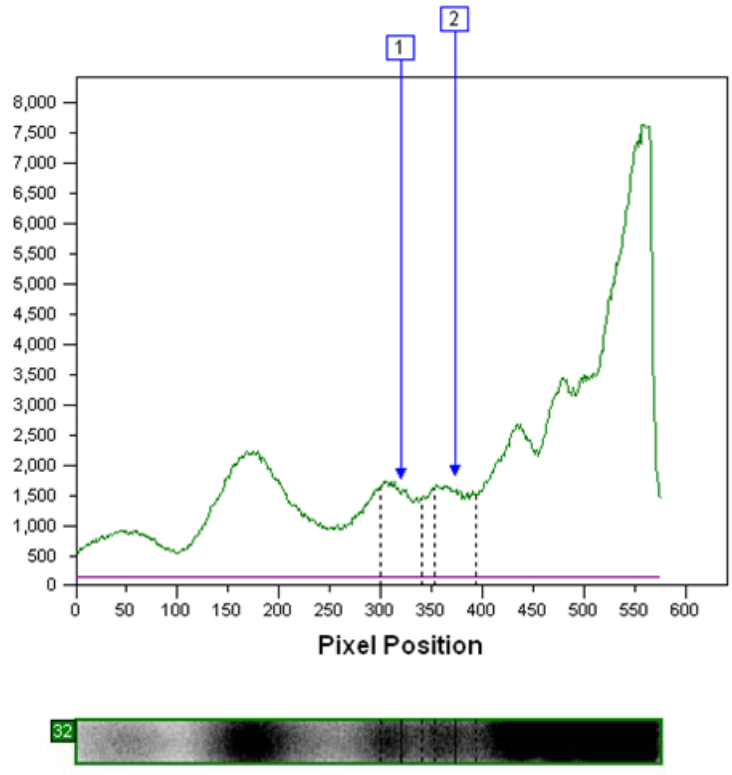


Figure 19 – Densitometry of a lane of a CFCA reaction corresponding to CePrP seeded with CWD. Bands, clearly detectable on the gel lane (bottom) were demarcated and the signal intensity was measured (upper trace). By comparison of such measurements to those for starting substrate, the percentage of converted product can be calculated.

omitted the positive control reaction for this seed. Additionally, we have not included any errors associated with these measurements since these are problematic to calculate with any accuracy given the calculations that were done to normalise data. It should be assumed, for the purposes of comparison, that error rates may be as high as ± 20 % of the value quoted.

Conversion efficiencies in %		**Cattle BSE**	**Ovine BSE**	**Ovine atypical scrapie**	**Cattle BASE**	**CWD**
Recombinant protein	**BovPrP**	7.0 (7.6)	NT	NT	NT	NT
	CePrP	NT	NT	NT	NT	4.5 (3.8)
	CePrP_E220Q	NT	NT	NT	NT	0 (0.2)
	OvPrP_ARQ	2.8 (6.3)	2.7 (6.1)	NT	NT	NT
	OvPrP_AHQ	NT	NT	0.7 (0.5)	NT	NT
	OvPrP_ARQ_L141F	0 (3.8)	NT	NT	NT	NT
	HuPrP_M129	4.5 (6.8)	2.6 (0.8)	1.9 (0.8)	3.8 (0.8)	2.9 (1.6)
	HuPrP_V129	0 (0.6)	0 (1.1)	0.4 (1.1)	1.9 (3.9)	0.8 (1.0)
	HuPrP_M129 / HuPrP_V129 1:1 mix	4.7 (6.9)	3.4 (6.1)	2.0 (6.9)	6.3 (9.7)	3.3 (7.6)

5. Discussion

This project set out to determine the likely transmission efficiencies of novel TSE isolates to humans, by means of cell free conversion assays. We sourced brain materials infected with atypical scrapie, atypical BSE (BASE) and chronic wasting disease and also used tissues from a sheep experimentally infected with BSE. In the absence of vCJD samples, we used cattle BSE as an independent control with which to seed assays. The lack of a homologous conversion system using human proteins is not ideal but does not represent major issues in interpretation of results. Such a system would not be useful for generation of data relevant to risk, since the *in vivo* transmission efficiency of vCJD between humans is completely unknown. Besides, positive conversions of HuPrP_M129 demonstrate the ability of this protein to convert and we have no reason to believe that the HuPrP_V129 variant is not capable of conversion. Previous CFCA-based assays have monitored the efficacy of the human-human system using vCJD and found low level, but detectable, conversion, which was more prevalent for M129-containing human protein than V129-containing protein[20]. It is more useful to relate conversion assays involving human substrates to a system with real disease transmission data that can be estimated – although unproven, cattle BSE is believed to have caused the vCJD epidemic in humans in the UK, although it is conceivable that infection may have passed indirectly by way of an intermediate disease-carrier, such as sheep. Interestingly, transmission of cattle and sheep BSE to humanised transgenic mice partially support this hypothesis (Barron, Andreoletti, personal communications). Nevertheless, it can be estimated that at least half the population was exposed to BSE-infected tissues but only ~200 cases resulted. Thus, the efficiency of disease transmission can be demonstrated to be low and associated with a small but definite risk.

Since we were performing cross species disease transmission, we anticipated that the levels of conversion would be low. Additionally, unlike other CFCA systems, our conversion assay buffer contains no denaturants[14], which we believe makes this a more physiological experimental system with which to test cross species disease transmission[13]. However, it does result in conversion efficiencies that are lower than previously reported[18]. For these reasons we wanted to develop an alternative method of detection of conversion assay products; traditionally the CFCA uses radiolabelled protein as a substrate and newly formed PK-resistant protein can be detected by autoradiography after Western blotting. We noted technology to allow the incorporation of unnatural amino acids site-specifically into proteins during expression in *E.coli* and, subsequently, such unnatural amino acids could be reacted specifically with particular fluorophores. This would result in recombinant protein that was labelled site specifically with a single fluorescent moiety. Such a technology is seductive,

because it would carry a range of side benefits aside from increases in sensitivity of detection. Fluorescence-based detection is substantially less hazardous that radioactivity, and does not diminish with time. It would also allow differential detection of two different proteins in a mixture, making available true CFCA experiments that mimic PRNP heterozygosity.

With help from the group of Schultz, we produced recombinant prion protein variants that incoporated the unnatural amino acid p-acetyl phenylalanine site specifically. To the free ketone group generated, we reacted a range of different fluorescent derivatives, which produced proteins that could be detected by fluorescence scanning of SDS-PAGE gels. However, we encountered severe problems during the final workup stages of fluorescent protein production – the fluorescently labelled protein adhered strongly to plasticware and was poorly soluble. Nevertheless, we produced data from conversion assay experiments that demonstrated the feasibility of fluorescence based conversion assays. The data also demonstrated that differential detection of two proteins from a mixture is possible. However, our methods to remove free, unreacted fluorophore, from the labelled protein were sub optimal in these initial experiments and resulted in the generation of additional labelled proteins (most probably PrP^{Sc}) during CFCA reactions, thereby complicating the analysis. We trialled a range of methods to remove free fluorophore but each resulted in loss of protein or generation of a protein that was no longer amenable to conversion. Although we have several other ideas for methods to remove free fluorophore and prevent non-specific reactions with other proteins in CFCA assays, we made the decision to use the traditional, radio-labelling approach for conversion assay work in this project.

The fluorescent tagging of recPrP remains a future goal of research within the group and experiments are ongoing. This would represent a means of detecting recPrP in various systems, not just in CFCA. We have current methods to generate fibrils of recPrP by means of shaking and the inclusion of fluorescently labelled protein in such techniques would allow the detection of fibrils by fluorescence microscopy. Fluorescence detection is a method that is used extensively to track proteins in *in vivo* experimentation and the use of site-specifically tagged reagents would augment and enhance such experiments immeasurably. We would also still like to use the fluorescently tagged proteins in CFCA experiments that mimic heterozygosity, since there exist many unknowns relating to TSE disease in animals that express two different prion proteins. In some cases, two different forms of the protein appear to decrease disease incubation time, whilst in others a dominant negative phenotype is

observed resulting in increase incubation times and reduced susceptibility to disease. Understanding such intricacies would aid the search for prion diagnostics and therapeutics.

Using our standard protocols for radiolabelling recPrP, we produced 10 different recPrP proteins more or less simultaneously to produce proteins that had sufficient activity for use. Of these 8 proteins gave good levels of activity at protein concentrations that we could use. However, expression of P102L mutants of human protein did not produce adequate levels of protein and CFCA experiments incorporating these proteins could not be done. We are not clear why this is, but we know from previous experiments that different recPrP molecules express at different rates and, therefore, incorporate different levels of radioactive methionine. Ovine proteins have represented continual problems in this regard, but we note that expression of ovine proteins in this project was successful. Cell free conversion assays were set up using the 8 forms of recPrP that we were able to express. We also included both positive and negative controls in all assays. After SDS-PAGE of the products, we visualised PK-resistant recombinant PrP isoforms by phosphorimaging and used densitometric methods to quantify levels of conversion.

Because of the low levels of conversion detected, and the necessity to use lower levels of PK than normal to accommodate atypical TSE strains, we found identification of the relevant PK resistant bands challenging. In some cases, positive control reactions had PK resistant bands of the appropriate molecular weight, whilst in others a higher molecular weight band was present. On the whole, intensity of the two bands appeared to be related such that a stronger lower band correlated with a stronger higher band also. In order to generate comparable data that was reliable, we have quantified conversion assuming that either band was the correct product. We therefore generated two sets of data, which are actually rather comparable in most cases. We are not sure what the upper band represents, but it is most probably aggregated species of PK-resistant protein – we also found higher molecular weight forms (higher even that the starting material) hence some form of aggregation must have occurred during CFCA incubations.

In common with previous reports, we found predominately that conversion efficiencies cross species were low. Conversion efficiencies in our murine system (seeded with either 87V or ME7) are of the order of 20% or greater. In contrast, we found reasonable but low levels of conversion using homologous conversion systems, although not all gave high levels of conversion. Cattle BSE, ovine BSE and CWD gave relatively strong homologous conversion, whilst atypical scrapie gave poor levels of homologous conversion. We did not

test BASE because of the low amount of tissue available. Variable homologous conversion is in line with experiments previously performed using mouse-passaged strains of scrapie to convert murine PrP. In such experiments, some scrapie strains result in efficient conversion with high levels of PK-resistant recPrP produced, whilst others result in low levels of conversion that are barely detectable. This is also the case for some forms of sheep scrapie. We are not clear why this is, either for murine systems or for those systems herein that result in low levels of conversion of positive controls. At a fundamental level, the low levels of conversion associated with a given scrapie strain would suggest low levels of susceptibility of animals to that strain, but we know from experimental transmissions in the target species that this is not always replicated. For example, ovine atypical scrapie appears to convert ovine protein rather poorly, implying that sheep would be resistant to infection with atypical scrapie, but positive experimental transmissions have been reported in both sheep and ovinised transgenic mice[26-28]. Conversely, atypical scrapie isolates convert human protein more readily suggesting a greater level of susceptibility of humans than sheep. However, it is important to remember that the cell free conversion assay represents a purified, idealised system in which to study the interaction between proteins. The system does not include cellular machinery (as discussed below) and has been optimised to produce conversion efficiencies that are a high as possible whilst retaining as much biological information as possible. Whilst the system is not 'forced' through the addition of exogenous sources of energy (sonication or shaking) it probably represents the maximum conversion that would result from interaction of proteins under physiological conditions. There will also be additional factors that limit the *actual* levels of conversion *in vivo*. On this basis, the only way that one can interpret data with any degree of confidence is by comparison across different strains of disease – i.e. comparing the conversion of human proteins by multiple different TSE isolates. The positive control homologous conversion assays (in these kind of experiments) should be viewed only as a demonstration that fibrils are present that are capable of seeded conversion reactions and should not be treated quantitatively.

With the human proteins tested, we found, in general, that M129-containing human protein converted significantly more readily than V129-containing protein. In mixtures of these proteins, to represent heterozygosity, levels were intermediate suggesting that the V129 allele does not have a dominant negative effect. Conversions with V129-containing protein were so low as to be negligible in the majority of cases. In terms of order of conversion, in the M129-containing protein experiments we found highest levels of conversion associated with assays seeded with cattle BSE. This is in line with the known transmission of BSE from cattle to humans carrying a methionine at PRNP codon 129. We

found that BASE converted almost as well as cattle BSE and in the heterozygous assays this order was reversed. BASE was also the only isolate that convert v129-containing human protein. This suggests similar conversion efficiencies from the two isolates that infect cattle. In M129-containing conversion assays, the other 3 isolates converted with lower efficiencies than cattle isolates and the efficiencies were broadly comparable. This was also the case for conversion systems in which a mixture of substrates was used. Taken together, these data suggest that BSE in sheep, atypical scrapie and CWD are less efficient at converting PrP than is cattle BSE or BASE.

Interpretation of these results in terms of risk assessment is somewhat difficult in the absence of comparable data from other experiments. We note that conversion assays are, by their nature, cell free and therefore mimic only certain aspects of TSE disease. These aspects are likely to be restricted to biochemical mechanisms that involve direct interactions between endogenous PrP^{C} and exogenous PrP^{Sc}, i.e. the conversion process at the very start of a TSE infection. In some cases, it may be that the levels of conversion at this time are what ultimately determine the overall susceptibility of an animal/human. However, there are inevitably going to be other processes occurring *in vivo* that are not replicated in a cell free system and a good example is that of clearance of protein aggregates. Such a process is implicated from various strands of TSE research and this will play a role in mitigating the initial infection as well as reducing speed or location of the spread of infection within an individual. To model these processes requires use of transgenic animal experiments. Thus, whilst our data will probably put an upper limit on the likely susceptibility of humans to novel TSE isolates (i.e. humans are not likely to be more susceptible to novel TSE isolates than they were to BSE) it is likely to over estimate rather than underestimate the likely transmission.

6. Conclusions

The overall conclusions of this project are that conversion efficiency of human proteins seeded with novel isolates of TSE disease are low and are not greater than that of control reactions seeded with cattle BSE. These data should be used in conjunction with equivalent data from experiments based around infection of transgenic mice. For future studies, similar cell free conversion assays systems could be used, but without additional mechanistic studies at biological levels between the cell free models and animal models, results will always be difficult to interpret. Cell culture models may provide a link in between these two very different paradigms.

We are continuing to work on the cell free conversion assays detailed within this project. We are still trying to optimise fluorescence labelling. We are also in the process of adding additional replicates to the conversion assay data that we have already generated. We will communicate these data to FSA as they are generated.

7. References

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8. Appendices

Appendix A – Recombinant PrP sequences used in this work

In all cases, differences between multiple sequences for a species are shown in bold

Recombinant Bovine PrP - BovPrP

MKKRPKPGGG WNTGGSRYPG QGSPGGNRYP PQGGGGWGQP HGGGWGQPHG GGWGQPHGGG
WGQPHGGGWG QPHGGGGWGQ GGTHGQWNKP SKPKTNMKHV AGAAAAGAVV GGLGGYMLGS
AMSRPLIHFG SDYEDRYYRE NMHRYPNQVY YRPVDQYSNQ NNFVHDCVNI TVKEHTVTTT
TKGENFTETD IKMMERVVEQ MCITQYQRES QAYYQRGA

Recombinant Deer PrP (1) - CePrP

MKKRPKPGGG WNTGGSRYPG QGSPGGNRYP PQGGGGWGQP HGGGWGQPHG GGWGQPHGGG
WGQPHGGGGW GQGGTHSQWN KPSKPKTNMK HVAGAAAAGA VVGGLGGYML GSAMSRPLIH
FGNDYEDRYY RENMYRYPNQ VYYRPVDQYN NQNTFVHDCV NITVKQHTVT TTTKGENFTE
TDIKMMERVV EQMCITQYQR ES**Q**AYYQRGA

Recombinant Deer PrP (2) – CePrP_Q213E

MKKRPKPGGG WNTGGSRYPG QGSPGGNRYP PQGGGGWGQP HGGGWGQPHG GGWGQPHGGG
WGQPHGGGGW GQGGTHSQWN KPSKPKTNMK HVAGAAAAGA VVGGLGGYML GSAMSRPLIH
FGNDYEDRYY RENMYRYPNQ VYYRPVDQYN NQNTFVHDCV NITVKQHTVT TTTKGENFTE
TDIKMMERVV EQMCITQYQR ES**E**AYYQRGA

Recombinant Human PrP – HuPrP_M129

MKKRPKPGGW NTGGSRYPGQ GSPGGNRYPP QGGGGWGQPH GGGWGQPHGG GWGQPHGGGW
GQPHGGGWGQ GGGTHSQWNK **P**SKPKTNMKH MAGAAAAGAV VGGLGGY**M**LG SAMSRPIIHF
GSDYEDRYYR ENMHRYPNQV YYRPMDEYSN QNNFVHDCVN ITIKQHTVTT TTKGENFTET
DVKMMERVVE QMCITQYERE SQAYYQRGS

Recombinant Human PrP – HuPrP_V129

MKKRPKPGGW NTGGSRYPGQ GSPGGNRYPP QGGGGWGQPH GGGWGQPHGG GWGQPHGGGW
GQPHGGGWGQ GGGTHSQWNK **P**SKPKTNMKH MAGAAAAGAV VGGLGGY**V**LG SAMSRPIIHF
GSDYEDRYYR ENMHRYPNQV YYRPMDEYSN QNNFVHDCVN ITIKQHTVTT TTKGENFTET
DVKMMERVVE QMCITQYERE SQAYYQRGS

Recombinant Human PrP – HuPrP_M129_P102L

MKKRPKPGGW NTGGSRYPGQ GSPGGNRYPP QGGGGWGQPH GGGWGQPHGG GWGQPHGGGW
GQPHGGGWGQ GGGTHSQWNK **L**SKPKTNMKH MAGAAAAGAV VGGLGGY**M**LG SAMSRPIIHF
GSDYEDRYYR ENMHRYPNQV YYRPMDEYSN QNNFVHDCVN ITIKQHTVTT TTKGENFTET
DVKMMERVVE QMCITQYERE SQAYYQRGS

Recombinant Human PrP – HuPrP_V129_P102L

MKKRPKPGGW NTGGSRYPGQ GSPGGNRYPP QGGGGWGQPH GGGWGQPHGG GWGQPHGGGW
GQPHGGGWGQ GGGTHSQWNK **L**SKPKTNMKH MAGAAAAGAV VGGLGGY**V**LG SAMSRPIIHF
GSDYEDRYYR ENMHRYPNQV YYRPMDEYSN QNNFVHDCVN ITIKQHTVTT TTKGENFTET
DVKMMERVVE QMCITQYERE SQAYYQRGS

Recombinant Ovine PrP – OvPrP_ARQ

MKKRPKPGGG WNTGGSRYPG QGSPGGNRYP PQGGGGWGQP HGGGWGQPHG GGWGQPHGGG
WGQPHGGGGW GQGGSHSQWN KPSKPKTNMK HVAGAAAAGA VVGGLGGYML GSAMSRP**L**IH
FGNDYEDRYY **R**ENMYRYPNQ VYYRPVDQYS NQNNFVHDCV NITVKQHTVT TTTKGENFTE
TDIKIMERVV EQMCITQYQR ESQAYYQRGA

Recombinant Ovine PrP – OvPrP_AHQ

MKKRPKPGGG WNTGGSRYPG QGSPGGNRYP PQGGGGWGQP HGGGWGQPHG GGWGQPHGGG
WGQPHGGGGW GQGGSHSQWN KPSKPKTNMK HVAGAAAAGA VVGGLGGYML GSAMSRP**L**IH
FGNDYEDRYY **H**ENMYRYPNQ VYYRPVDQYS NQNNFVHDCV NITVKQHTVT TTTKGENFTE
TDIKIMERVV EQMCITQYQR ESQAYYQRGA

Recombinant Ovine PrP – OvPrP_ARQ_L141F

MKKRPKPGGG WNTGGSRYPG QGSPGGNRYP PQGGGGWGQP HGGGWGQPHG GGWGQPHGGG
WGQPHGGGGW GQGGSHSQWN KPSKPKTNMK HVAGAAAAGA VVGGLGGYML GSAMSRP**F**IH
FGNDYEDRYY **R**ENMYRYPNQ VYYRPVDQYS NQNNFVHDCV NITVKQHTVT TTTKGENFTE
TDIKIMERVV EQMCITQYQR ESQAYYQRGA

Recombinant Ovine PrP – 201TAG – asterisk is the TAG amber stop codon

MKKRPKPGGG WNTGGSRYPG QGSPGGNRYP PQGGGGWGQP HGGGWGQPHG GGWGQPHGGG
WGQPHGGGGW GQGGSHSQWN KPSKPKTNMK HVAGAAAAGA VVGGLGGYML GSAMSRPLIH
FGNDYEDRYY RENMYRYPNQ VYYRPVDQYS NQNNFVHDCV NITVKQHTVT TTTKGEN*TE
TDIKIMERVV EQMCITQYQR ESQAYYQRGA

Recombinant Ovine PrP – 234TAG – asterisk is the TAG amber stop codon

MKKRPKPGGG WNTGGSRYPG QGSPGGNRYP PQGGGGWGQP HGGGWGQPHG GGWGQPHGGG
WGQPHGGGGW GQGGSHSQWN KPSKPKTNMK HVAGAAAAGA VVGGLGGYML GSAMSRPLIH
FGNDYEDRYY RENMYRYPNQ VYYRPVDQYS NQNNFVHDCV NITVKQHTVT TTTKGENFTE
TDIKIMERVV EQMCITQYQR ESQAYYQRGA*

Recombinant Murine PrP – 197TAG – asterisk is the TAG amber stop codon

MKKRPKPGGW NTGGSRYPGQ GSPGGNRYPP QGGTWGQPHG GGWGQPHGGS WGQPHGGSWG
QPHGGGWGQG GGTHNQWNKP SKPKTNLKHV AGAAAAGAVV GGLGGYMLGS AMSRPMIHFG
NDWEDRYYRE NMYRYPNQVY YRPVDQYSNQ NNFVHDCVNI TIKQHTVTTT TKGEN*TETD
VKMMERVVEQ MCVTQYQKES QAYYDGRRS