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Screening for vCJD infectivity in blood

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Why do we need a screening assay?

- **The infectious agent responsible for vCJD can be transmitted through blood transfusion and possibly through contaminated plasma products.**
- **Blood from affected individuals is infectious during a protracted asymptomatic, pre-clinical disease phase.**
- **Concern that pool of infectious, asymptomatic individuals could be routinely donating blood leading to further cases of secondary disease transmission.**

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vCJD and blood – What we don't know

- **The true prevalence of pre-clinical disease in the UK donor population.**
(Current estimate that 1/4000 may be incubating pre-clinical disease)
- **Nature, distribution and level of infectivity in human blood.**
We have to rely on assumptions made from animal models.
- **At what time point during disease progression does blood become infectious?**

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Development of a diagnostic/screening assay

- **No disease associated detectable immune response and no disease associated DNA/RNA.**
- **Animal infectivity bioassays.**
(Expensive, can take years to get results, results not always conclusive)
- **Use of surrogate markers.**
- **Key event in prion disease pathogenesis is thought to be the conversion of normal cellular prion protein (PrP^{C}) to a misfolded, aggregated form termed PrP^{Sc} and PrP^{Sc} is the only unambiguous disease marker identified to date**
- **Most screening assays under development rely on the detection of PrP^{Sc} .**
- **Relationship between PrP^{Sc} and infectivity is complex!**

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Requirements for an assay capable of detecting PrP^{Sc} in blood

The assay will need to:-

- Detect minute amounts (possibly as low as ag/ml amounts) of PrP^{Sc} in a large excess (~100ng/ml) of PrP^C.
- Not be influenced by the matrix (whole blood/plasma/buffy coat/WBC).
- Be highly specific to avoid large numbers of false positive results.
- Ideally two type of assay are required:-
 - A rapid high-throughput assay to routinely screen all blood donations.**
 - A confirmatory assay to ensure that all positive results from the initial screening assay are indeed true positives.**

Methods of distinguishing between PrP^C and PrP^{Sc}

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- **Proteinase K (PK) resistance**
Limited digestion with proteinase K completely degrades PrP^C leaving the PrP^{Sc} PK resistant core (PrP^{res}) intact.
PK sensitive forms of PrP^{Sc} implicated in disease pathogenesis so ideally need to avoid using PK.
- **Epitope masking during the of conversion of PrP^C to PrP^{Sc}.**
Conformation Dependent Immunoassay
Amorfix Epitope Protection Assay
- **PrP^{Sc} specific antibodies**
Bind to epitopes exposed/created during the conversion of PrP^C to PrP^{Sc}.
Prionics assay
- **PrP^{Sc} specific ligands**
(plasminogen, synthetic polymers)
- **Reagents that selectively precipitate PrP^{Sc}.**
(Phosphotungstic acid, streptomycin)

Based on the UK Blood Transfusion Services recommendations a screening assay needs to meet the following criteria:-

- **Can specifically and reproducibly detect vCJD PrP^{Sc} down to the sensitivity predicted to be required for the detection of infectivity in the blood of individuals incubating pre-clinical disease.**

Based on recommendations from the CJD Resource Centre Oversight Committee an acceptable assay should reproducibly detect PrP^{Sc} in plasma samples spiked with between 10⁻⁴ to 10⁻⁷ dilutions of 10% vCJD brain homogenate and 10⁻¹ to 10⁻² dilutions of 10% vCJD spleen homogenate with no signal above the cut-off for any sample spiked with normal brain/spleen.

- **Have a low false positive rate (specificity of 99.85%) when large numbers (>5000) of normal blood samples are screened.**
- **Can specifically detect PrP^{Sc} in clinical/pre-clinical blood using a suitable animal model.**
- **Can specifically detect PrP^{Sc} in the blood of individuals with confirmed clinical vCJD.**

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Rapid, high-throughput screening assays

Two lead candidates:-

Amorfix Epitope Protection Assay (EP-CJD)

Prionics Check vCJD 3.2 assay

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Amorfix Epitope Protection Assay (EP-CJD)

96-well plate immunoassay based on the fact that during the conversion of PrP^C to PrP^{Sc} certain epitopes (recognised by anti-PrP antibodies) that would normally be exposed in native PrP^C become buried/trapped within the resulting misfolded PrP^{Sc} aggregates.

1ml citrated plasma



PrP^{Sc} concentration step



Sample treated with peroxynitrite (a strong oxidizing agent) that modifies amino acids so that all epitopes exposed in native PrP^C and on the surface of native PrP^{Sc} aggregates are destroyed, whereas, those epitopes buried within the PrP^{Sc} are protected



Denaturation of the peroxynitrite treated samples to expose protected epitopes



Detection of exposed epitopes using suitably labelled antibodies.

Two 96-well microplates (160 test samples) can be processed within 4 hours

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Outcome of the EP-CJD Assay

- **Could reproducibly detect PrP^{Sc} in plasma spiked with a 10⁻⁵ dilution of a 10% vCJD brain homogenate.**
- **Low false positive rate (specificity of 99.95%) when large numbers (39,000) of normal plasma samples were screened.**
- **Using animal models could detect PrP^{Sc} in clinical/pre-clinical plasma. (BSE in primates and scrapie in sheep)**
- **Failed to detect PrP^{Sc} in plasma from individuals with confirmed clinical vCJD.**
- **At this point Amorfix decided to stop all further work on assay development!**

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Prionics Check vCJD 3.2 assay

96-well plate based sandwich immunoassay based on the use of a PrP^{Sc} specific antibody(15B3) as the capture antibody.

Plasma filtered through a 96-well filter plate



Bound PrP^{Sc} is eluted directly into 96-well plate coated with 15B3



PrP^{Sc} captured by 15B3 is separated from PrP^C by washing



PrP^{Sc} conditioned for detection



Bound PrP^{Sc} detected by chemiluminescence using HRP anti-PrP antibody

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Prionics Check vCJD 3.2 assay

Progress with assay development to date

- **Can reproducibly detect PrP^{Sc} in plasma spiked with a 10⁻⁵ dilution of a 10% vCJD brain homogenate.**
- **Diagnostic specificity (>3400 samples):- 98.74% based on initial reactives
99.97% based on repeat reactives**
- **Currently evaluating the assay using plasma from an animal model.
(blinded sheep scrapie plasma panel)**
- **Yet to screen large numbers of normal plasma samples.**
- **Yet to request access to clinical vCJD plasma samples.**

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Confirmatory screening assays

Assay based on the amplification of PrP^{Sc} to readily detectable levels by Protein Misfolding Cyclic Amplification (PMCA)

Assay being developed independently by research groups in both the UK (SNBTS/NCJDRSU) and France (EFS)

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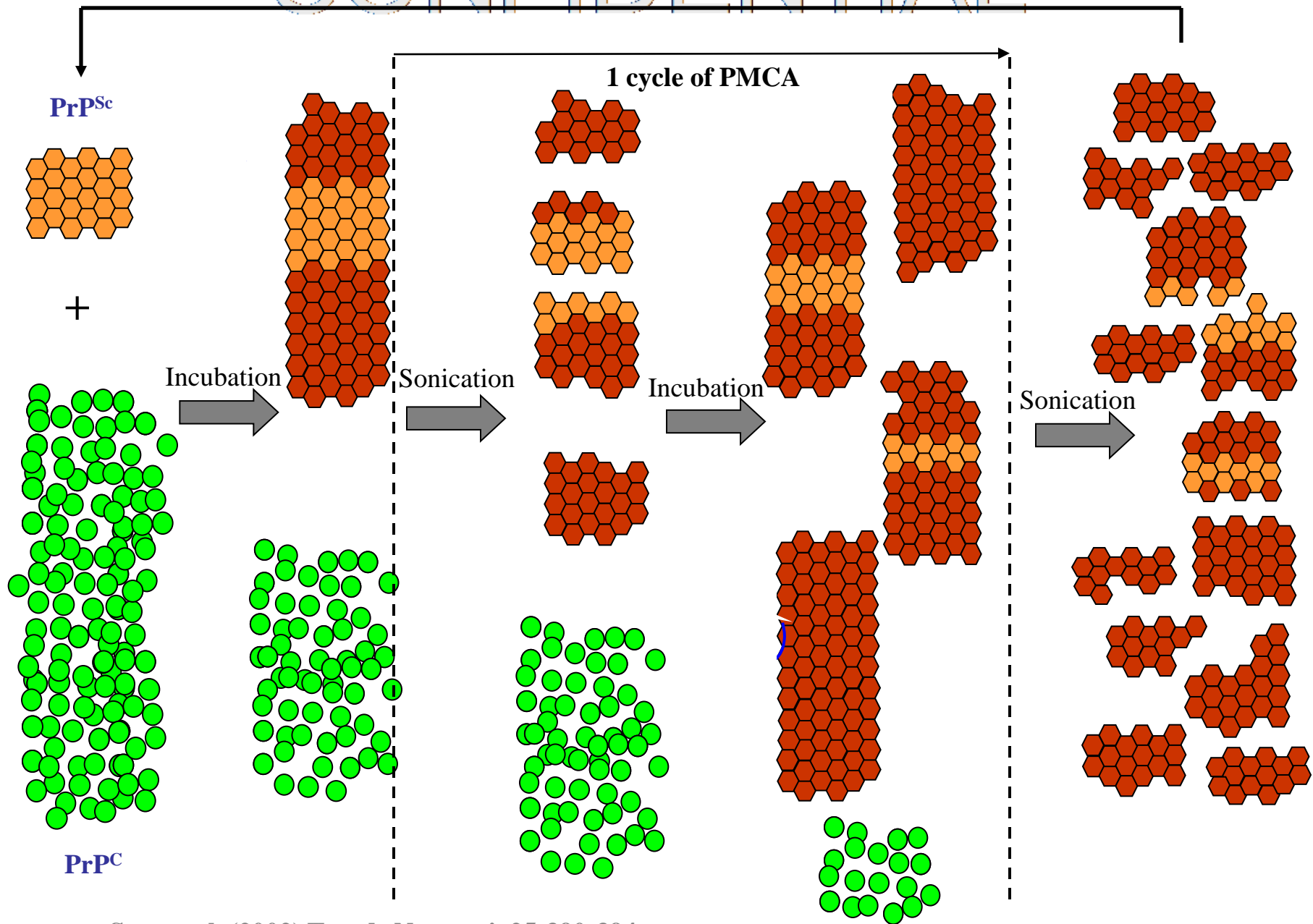
Protein misfolding cyclic amplification (PMCA)

***In vitro* conversion system aimed to rapidly replicate the autocatalytic protein misfolding cascade which is believed to be the core of prion pathogenesis.**

PMCA is not a PrP^{Sc} screening assay but simply a method of amplifying PrP^{Sc} to readily detectable levels which can be detected using a suitable PrP^{Sc} detection assay.

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Principle of PMCA



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Amplification of PrP^{Sc} associated with human prion diseases

- **Various substrates shown to support vCJD PrP^{Sc} amplification.**
(human brain tissue, *PRNP* humanised transgenic mouse brain tissue and human platelets)
- **By Western blotting following limited PK digestion amplified PrP^{Sc} shown to retain the molecular characteristics associated with the PrP^{Sc} seed.**
(PrP^{res} glycoform ratio and electrophoretic mobility)

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Problems associated with PMCA

- **Reproducibility of PrP^{Sc} amplification between individual tubes/wells in a single experiment, between experiments and between sonicators.**
- **Reports of de novo/spontaneous generation of PrP^{Sc} during sPMCA.**
- **For humans need to ensure seed/substrate *PRNP* codon 129 compatibility.**
Unless the *PRNP* codon 129 genotype of the test sample is known then the sample will have to be tested in both *PRNP*-129MM and *PRNP*-129VV substrate.
- **Human plasma contains factors which inhibit PMCA.**
Need plasma pre-treatment method to remove the inhibitory factors.

Comparison of the UK and French PMCA based methods

	UK Method (SNBTS/NCJDRSU)	French Method (EFS)
Volume of plasma used	180µl	500µl
Plasma pre-treatment (Concentration of P^{Sc}, removal of inhibitory factors)	NaCl precipitation	Capture of PrP^{Sc} by ligand coated beads
PMCA substrate [10% (w/v) homogenate]	Human platelets	<i>PRNP</i> humanised transgenic mouse (<i>tg650</i> overexpressing PrP^{PMET129}) brain tissue
Rounds of serial PMCA	4 rounds (48 cycles per round)	3 rounds (80 cycles per round)
PrP^{Sc} detection method	Conformation dependent immunoassay (CDI)	Detection of PrP^{Pres} by Western blotting following limited proteinase K digestion
PrP^{Sc} detection sensitivity in spiked plasma	10⁻⁸ and 10⁻⁵ dilutions of 10% (w/v) vCJD brain and vCJD spleen homogenates respectively	10⁻⁹ dilution of a 10% (w/v) vCJD brain homogenate
Specificity	Control plasma spiked with non- CJD brain/spleen homogenate and 20 normal plasma samples supplied by NIBSC all tested negative.	20 normal plasma samples supplied by NIBSC all tested negative

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Conformation dependent immunoassay (CDI)

96-well DELFIA based sandwich immunoassay which relies on the use of an antibody (3F4) that binds to an epitope that is buried in native PrP^{Sc} only becoming exposed following denaturation.

Phosphotungstic acid (NaPTA) precipitation to selectively precipitate PrP^{Sc}



Resulting pellet resuspended and split into two equal lots with one lot retained in its native format (N sample) and the other lot denatured (D sample)



N and D samples are screened by sandwich DELFIA assay using Eu-labelled 3F4 as the detection antibody



TRF in the N and D samples is measured and the results expressed as the D/N ratio



Assay cut-off value calculated as the mean D/N ratio obtained for negative controls plus 3 standard deviations.



For a test samples any D/N ratio value above the cut-off is regarded as positive.

Aims of the project

- **Development of an optimal, reproducible PMCA based confirmatory screening assay based on the best combination of:-**
 - Plasma pre-treatment method** (four methods being evaluated)
 - PMCA substrate** (human platelets vs tg650 mouse brain)
 - sPMCA method** (4 rounds of 48 cycles/round vs 3 rounds of 80 cycles/round)
 - PrP^{Sc} detection method** (CDI vs Western blotting following limited PK digestion)
- **Does amplification of PrP^{Sc} also result in the amplification of infectivity?**
(Bioassay in tg650 mice)

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Recent developments in vCJD screening

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Lancet 2011 Feb 5; 377(9764): 487-93.

Detection of prion infection in variant Creutzfeldt-Jakob disease: a blood-based assay.

Edgeworth JA, Farmer M, Sicilia, Tavares P, Beck J, Campbell T, Lowe J, Mead S, Rudge P, Collinge J, Jackson GS.

MRC Prion Unit, Department of Neurodegenerative Disease, UCL Institute of Neurology, London, UK.

MRC Prion Unit whole blood screening assay

- **Assay based on the selective binding of PrP^{Sc} to stainless steel particles followed by immunodetection using a suitably labelled anti-PrP monoclonal antibody.**
- **Able to detect PrP^{Sc} from whole blood (400µl) spiked with a 10⁻¹⁰ dilution of vCJD brain homogenate.**
- **Each test sample tested twice with only samples reactive in both tests classed as positive**
- **Reported sensitivity of 71.4% and specificity of 100% based on the limited numbers whole blood samples tested.**

vCJD: 15/21 were reactive in both tests and classed as positive.

(3/6 negatives were reactive in one but not both tests)

Normal: 92/100 negative in both tests and 8 were reactive in one but not both tests.

sCJD: 16/16 negative in both tests.

Other neurological disorders: 42/42 negative in both tests.

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MBio. 2011 May 10;2(3). pii: e00078-11. doi: 10.1128/mBio.00078-11. Print 2011.

Prion disease blood test using immunoprecipitation and improved quaking-induced conversion.

Orrú CD, Wilham JM, Raymond L, Kuhn F, Schroeder B, Raeber AJ, Caughey B.

Laboratory of Persistent Viral Diseases, Rocky Mountain Laboratories, National Institute of Allergy and Infectious Diseases, National Institutes of Health, Hamilton, Montana, USA.

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Enhanced Quaking-Induced Conversion (eQuIC)

- **Developed from a format called Real-time QuIC (RT-QuIC).**
- **Multi-well plate based assay employing Thioflavin T based fluorescence detection of amyloid fibrils formed when a reaction mixture containing recombinant prion protein (recPrP) is seeded with PrP^{Sc}.**
- **RT-QuIC shown to have a >80% sensitivity and 100% specificity in discriminating between sCJD and non-CJD patients based on testing CSF samples.
(Hamster recPrP used as a substrate)**
- **The vCJD problem.
(vCJD samples did not appear to work in RT-QuIC experiments)**
- **Plasma contains inhibitors which prevent seeded amyloid formation when plasma is added directly into the reaction mixture.**
- **Introduction of a PrP^{Sc} immunoprecipitation step to capture PrP^{Sc} and remove the reaction inhibitors and the use of a chimeric hamster/sheep recPrP substrate**

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eQuIC assay protocol

Spin down plasma sample (500 μ l) at 16,000 RCF for 30sec



Capture PrP^{Sc} using magnetic beads coated with 15B3 (overnight at RT), pull down beads using a magnet, wash beads twice, resuspend beads in a 20 μ l final volume



Seed reaction mixture (100 μ l) with 4 μ l resuspended beads (four replicate reactions)
(chimeric hamster/sheep recPrP + Thioflavin T)

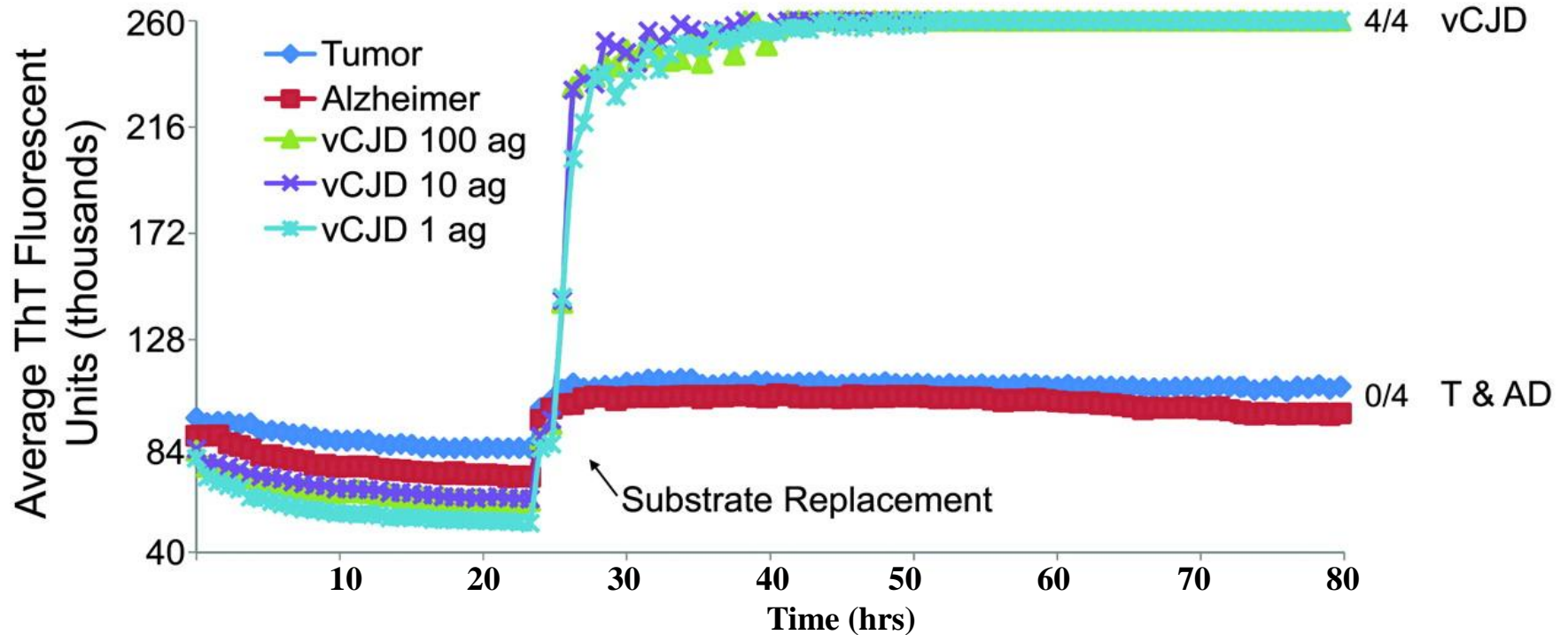


Incubate with shaking (1min on/1min off) at 46°C measuring fluorescence every 15min



After 24hr replace reaction mixture and continue QUIC for a further 36 – 60hrs

eQuIC detection of human PrP^CJD spiked into human plasma.



Dilutions of human non-prion (tumor and Alzheimer's disease) control or vCJD brain tissues were spiked into 500 μ l of human plasma to give final dilutions of 4×10^{-7} (tumor and Alzheimer's disease) and 4×10^{-12} , 4×10^{-13} , and 4×10^{-14} (vCJD; containing ~ 100 , 10, and 1 ag PrPres, respectively). PrP^CJD was immunoprecipitated with 15B3-coated beads and a portion of the beads were used to seed replicate eQuIC reaction mixtures containing 400 mM NaCl. After 24 h, the substrate was replaced. Chimeric Ha-S rPrP^C was used as a substrate in all reactions. The vertical axes indicate the average fluorescence from four replicate wells, and the fractions on the right indicate the positive/total replicate reactions associated with the adjacent traces.