TECHNICAL REPORT

Laboratory standard operating procedure for detecting sporadic Creutzfeldt-Jakob disease using Real-Time Quaking-Induced Conversion (RT-QuIC) assay

Version 1.0

February 2021

www.ecdc.europa.eu

ECDC TECHNICAL REPORT

Laboratory standard operating procedure for detecting sporadic Creutzfeldt-Jakob disease using Real-Time Quaking-Induced Conversion (RT-QuIC) assay

Version 1.0 February 2021

This report was commissioned by the European Centre for Disease Prevention and Control (ECDC) and produced by the National CJD Research and Surveillance Unit (NCJDRSU), University of Edinburgh, United Kingdom.

Authors
Neil McKenzie, Lynne McGuire and Alison Green at the National CJD Research and Surveillance Unit, University of Edinburgh, United Kingdom, EH4 2XU.

Suggested citation: European Centre for Disease Prevention and Control. Laboratory standard operating procedure for detecting sporadic Creuzfeldt-Jakob disease using Real-Time Quaking-Induced Conversion (RT-QuIC) assay. Stockholm: ECDC; 2021.

Stockholm, February 2021

ISBN 978-92-9498-529-3
doi: 10.2900/271946
Catalogue number TQ-04-21-058-EN-N

Contents

Tables

Abbreviations

Abs_{280}	Absorbance at 280 nm wavelength
bp	(DNA) base pair
BH	Brain homogenate
CJD	Creutzfeldt-Jakob disease
CSF	Cerebrospinal fluid
dsDNA	Double-stranded DNA
E. coli	*Escherichia coli*
EDTA	Ethylenediaminetetraacetic acid
FPLC	Fast Protein Liquid Chromatography
Ha	Hamster derived
Ham FL	Hamster full length (residues 23-231)
Ham-Sh	Hamster – sheep chimeric protein
Hu	Human derived
Hum FL	Human full length
IBs	Inclusion Bodies
IPTG	Isopropyl β-D-1-thiogalactopyranoside
LB	Lysogeny Broth
MBSC	Microbiological Safety Cabinet
MM1	Genotype that is homozygous for methionine at codon 129, Type 1
MWCO	Molecular-weight cut-off
Nickel-IMAC	Nickel Immobilized Metal Affinity Chromatography
NTA	Nitrilotriacetic acid
OETB	Overnight Express Instant TB Medium
o/n	Overnight
PBS	Phosphate-Buffered Saline
PrP	Prion Protein
PrP^{Sc}	Prion Protein (insoluble/disease causing/'scrapie' form)
PrPres	PK-resistant Prion Protein
PK	Proteinase K (a broad spectrum Serine protease)
RCF	Relative Centrifugal Force
recPrP	Recombinant Prion Protein
RFU	Relative fluorescence unit
RT-QuIC	Real-Time Quaking-Induced Conversion (assay)
sCJD	Sporadic Creutzfeldt-Jakob disease
SDS	Sodium Dodecyl Sulfate
TAE	Tris-Acetate-EDTA buffer
TB	Terrific Broth
ThT	Thioflavin T., a solvatochromic fluorescent dye
vCJD	Variant Creutzfeldt-Jakob disease
w/v	Weight to volume

Scope and principles

This document provides laboratories with a single protocol for producing recombinant full-length hamster prion protein and using it to perform the Real-Time Quaking-Induced Conversion assay (RT-QuIC), which can distinguish sporadic Creutzfeldt-Jakob disease (sCJD) from variant Creutzfeldt-Jakob disease (vCJD).

This protocol describes how to produce recombinant hamster prion protein and use it to perform RT-QuIC. It is based on methods published by the Caughey Group[1] and the National CJD Research and Surveillance Unit at the University of Edinburgh, United Kingdom (NCJDRSU) [2].

PrP expression and purification

Production of recombinant hamster Prion protein (Ha recPrP) uses standard techniques in molecular biology and affinity purification. The protein is expressed in bacteria, purified over a Nickel-IMAC column and then dialysed and concentrated. There are several steps during the purification which are critical to obtaining satisfactory yield, purity and function. These have been marked at the appropriate point in the procedural sections with the comment 'NB'.

The current protocol for expression of Ha recPrP requires use of the BL21 Rosetta DE3/pRSET system from Novagen. This is sold under license by Novagen and may not be freely shared between organisations. For this reason, although the Ha PrP gene can be supplied on an alternate plasmid, the gene must be excised and ligated into the correct bacterium and vector. Using the specified BL21 bacterium and plasmid provides good yields of high quality Ha recPrP. Use of other commercial bacterial expression systems may affect the final yield of protein but should give good quality Ha recPrP.

RT-QuIC assay

The Real-Time Quaking-Induced Conversion (RT-QuIC) assay is a protein aggregation assay. A buffered solution of recombinant PrP (recPrP) is prepared and then aliquoted out into a 96-well plate. The wells of the plate are then 'seeded' in quadruplicate with either a control sample or a test sample. If the added sample contains a PrP^{Sc} seed, it will cause the recPrP to aggregate and make the Thioflavin T (ThT) fluoresce.

In this assay, recPrP aggregation is promoted by shaking and heating in the presence of an sCJD seed. When protein, such as recPrP, aggregates in the presence of ThT, the ThT binds to the protein and the fluorescent properties of the dye change. The amount of aggregation can therefore be followed by exciting the ThT dye at 450 nm and recording the fluorescence emission at 480 nm.

The assay has 91% sensitivity and 98% specificity in detecting sCJD [3, 4]. It has been reported as being capable of detecting ≥ 1fg PrP^{Sc} in a sample [4]. The test is negative for vCJD.

In the RT-QuIC assay, the control samples used are brain homogenates and the test samples are cerebrospinal fluid (CSF). Both control and CSF samples are run in quadruplicate (i.e. four wells allocated on the plate per sample) to reduce the risk of false positives biasing the test outcome.

The control samples are:

- unseeded master mix.
- 100 fg of PrP from sudden-death brain.
- 100 fg of PrP from Alzheimer's diseased brain.
- 100 fg of sCJD PrPres from MM1 genotype brain.

Control samples may be obtained by petitioning a local neuropathology laboratory.

An example plate layout is shown in the appendix. Example traces showing the typical responses of controls and CSF samples are also included in the annexes.

For the control samples, 2 µL of brain homogenate is used to seed each quadruplicate well set. Of the four control types, only those wells spiked with sCJD brain homogenate exhibit a large increase in relative fluorescence unit (RFU) during the RT-QuIC assay. The RFU of the other control wells remain low and constant throughout the whole assay.

For the CSF test samples, 30 µL of CSF is used to seed each quadruplicate well set. CSFs are tested at 30 µL volumes in the first instance and are only tested further, at 15 µL and 7 µL, if the results of the 30 µL tests are equivocal. If a CSF sample contains a sCJD seed, the well exhibits an increase in RFU during the RT-QuIC assay. If the CSF sample does not contain a sCJD seed, the RFU remains low and constant throughout the whole assay.

Reagents

Table 1. Reagents for production of recombinant prion protein

Reagent
Acrylamide gels
Agarose
Antibiotic(s) appropriate for plasmid
BL21 DE3 Rosetta *E. coli* competent cells
BugBuster Master Mix
DNA loading dye
Dry ice or liquid nitrogen
Glycerol, sterile
Guanidine.HCl
Hydrochloric acid
Imidazole.HCl
Lysogeny broth
Molecular weight markers for DNA and protein gels
NTA Superflow Ni charged resin (500ml)
Overnight Express instant TB medium ('OETB' media, sufficient for 2L culture/purification run)
Plasmid containing the gene for Ha PrP
pRSET vector
Rapid DNA ligation kit
Restriction enzymes appropriate for excising the Ha PrP gene from the supplied plasmid
SOC medium
Sodium chloride
Sodium hydrogen phosphate (dibasic)
Sodium hydroxide
Sodium phosphate
TAE (Tris-Acetate-EDTA) buffer, 10x concentrate
Terrific broth
Tris
Water ice
Water, sterile

Table 2. Reagents for quantitating the amount of control seed used in the RT-QuIC Assay

Reagent	Concentration	
Proteinase K	50 mg/ml	
N2 supplement (Invitrogen)	1 x stock	
Recombinant Hu PrP (as reference)	50 – 200 fg/µL	
0.1% SDS in a 1 x PBS solution	0.1% SDS, 10 mM phosphate, 154 mM NaCl	
Anti-prion protein antibody 3F4 epitope	As specified by manufacturer	
Samples	**Amount**	**Source Location**
Sudden-death brain	approx. 100 mg	Frontal cortex
Alzheimer's diseased brain	approx. 100 mg	Frontal cortex
sCJD brain	approx. 100 mg	Frontal cortex

Table 3. Reagents for performing the RT-QuIC Assay

Reagent	Concentration	Volume per well
Hamster Prion Protein	0.3-0.5 mg/ml	20 – 30 µL (10 µg after spin filtration)
Phosphate Buffered Saline (PBS), 5x conc	50 mM phosphate 770 mM NaCl	20 µL
0.1% SDS in a 1 x PBS solution	10 mM Phosphate 154 mM NaCl 0.1% SDS	Not used in RT-QuIC assay, only used to determine recPrP concentration
Sodium chloride solution	2 M	8.5 µL
EDTA	100 mM	1 µL
ThT *(make 10 mM and dilute 10x)*	1 mM	1 µL
Water, molecular biology grade	-	(100-30.5-recPrP–sample) µL
Samples	**Concentration**	**Volume per well**
Sudden-death brain homogenate	50 fg Hu PrP/µL[†]	2 µL
Alzheimer's diseased brain homogenate	50 fg Hu PrP/µL[†]	2 µL
sCJD brain homogenate	50 fg Hu PrPres/µL[†]	2 µL
CSF sample	Neat	30, 15 or 7 µL

[†]Concentrations are determined using western blot. Quantitation is calculated as detailed in the section 'Preparing the brain homogenate controls'.

Prepare fresh reagent buffers as above and filter through a 0.22 µ filter. The hamster prion protein may be frozen and thawed, so long as it is spin-filtered through a 100 kDa molecular weight cut-off (MWCO) filter before use. After dilution into the master mix, the 1x PBS is pH 7.4.

N.B. The ThT should be made up at a 10 mM concentration (e.g. 32 mg ThT/10 ml water) to allow accurate weighing. The ThT should be solubilised at room temperature, then passed through a 0.22 µ filter. The yellow solution is stored in foil at 4°C until ready for use, at which point it is diluted ten-fold in water to afford a 1mM solution. This 10 mM master solution should be freshly made for every RT-QuIC assay.

Apparatus

Table 4. Apparatus for production of recombinant prion protein

Apparatus
+ 4 °C fridge or cold room
0.22 µ filter papers for degassing units and filtering solutions
0.22 µ filters with Luer lock syringe attachments
-20 °C freezer
-80 °C freezer
A packable FPLC column with >150 ml internal volume (e.g. XK 26/40 column)
Agar plates, impregnated with appropriate antibiotic
Amicon Ultra15 centrifugal units, 8 pk
Autoclave tape
Benchtop rocker/shaker/roller
Cell spreader
Centrifuge and rotor that can spin 6 x 50 ml Falcon tubes at 13 500 RCF
Containers for wet ice, dry ice, liquid nitrogen and dialysis
Cuvette(s) with UV-Vis transparency at 250 nm-700 nm
Dialysis tubing clips
Dialysis tubing with a 7 KDa MWCO
Disposable culture flasks, 2.5L, non-baffled
Disposable scalpels
DNA gel extraction kit
DNA ligase kit
DNA spin-purification kit
Eppendorfs
Equipment for running and visualising SDS-PAGE and agarose gels
Falcon tubes
Filter units for degassing buffers

Apparatus
FPLC equipment, including computer and control software
Glass bottles for storing sterile buffers, with capacities from 250 ml – 2 L
Incubator shaker with shaker plate and flask clamps
Measuring cylinders, capacities from 20 ml to 2 L
Metal foil
Microbiological safety cabinet Level 2 OR Bunson burner
Microfuge
Mini-prep kit
PCR tubes
Pipettes and sterile tips, ranging from 0.1 µL to 1 ml
Plastic pipettes,
Sharpsafe
Spatulas
Spectrophotometer (e.g. Nanodrop)
Syringes, polypropylene Luer lock tips, capacities from 1 ml–20 mL
UV-light box

Table 5. Apparatus for quantitating the amount of control seed used in the RT-QuIC Assay

Apparatus
Alkali resistant carboy (for disposal of samples, Eppendorfs and well plates)
Eppendorfs, 2 ml
Equipment and software for running, imaging and quantitating western blots
Equipment for running and visualising SDS-PAGE
Heating block
Level 2 Microbiological Safety Cabinet (MBSC) inside a Category 3 laboratory
Pipettes and sterile filter tips, ranging from 0.1 µL to 1 ml
Scalpel
Sharpsafe
Sterile filters, 0.22 µ porosity, with built-in Luer-lock screw attachment
Syringes, polypropylene Luer-lock tips, capacity 10 ml

Table 6. Apparatus for performing the RT-QuIC Assay

Apparatus
Alkali resistant carboy (for disposal of samples, Eppendorfs and well plates)
BMG Labtech FLUOstar Omega or Optima, including computer and control software
Clear polyolefin adhesive tape for sealing 96 well plate
Eppendorfs, 2 ml
Falcon tubes, 15 and 50 ml
Level 2 MBSC inside a Category 3 laboratory
Optical flat bottom plate, 96 well
Pipettes and sterile filter tips, ranging from 0.1 µL to 1 ml volume
Sharpsafe (for disposal of pipette tips)
Spin filtration units, with 100 kDa MWCO
Sterile filters, 0.22 µ porosity, with built-in Luer-lock screw attachment
Syringes, polypropylene Luer-lock tips, capacity 10 ml

Procedures

Production of RecPrP protein

Molecular biology

The cDNA used to express the Ha recPrP is based on PRNP from *Mesocricetus auratus* (Golden Syrian Hamster), NCBI Gene ID: 101829062. Residues 23-231 are expressed.

The gene sequence used for expression is:

5′-
atggcgaaccttagctactggctgctggcactctttgtggctatgtggactgatgttggcctctgcaagaagcggccaaagcctggagggtggaacactggcggaa
gccgataccctgggcagggcagccctggaggcaaccgttacccacctcagggtggcggcacatggggacagccccatggtggtggctggggtcagccccatggt
ggtggctggggtcaaccccatggtggtggctggggtcagccccatggtggtggctggggtcagggaggtggcacccacaatcagtggaacaagcccagtaagcc
aaaaaccaacatgaagcacatggccggcgctgctgcggcaggggccgtggtgggggccttggtggctacatgctggggagtgccatgagcaggcccatgatg
cattttggcaatgactgggaggaccgctactaccgtgaaaacatgaaccgctaccctaaccaagtgtattaccggccagtggaccagtacaacaaccagaacaact
ttgtgcacgattgtgtcaacatcaccatcaagcagcacacagtcaccaccaccaccaagggggagaacttcacggagaccgacatcaagataatggagcgcgtgg
tggagcagatgtgtaccacccagtatcagaaggagtcccaggcctactacgatggaagaaggtccagcgcggtgctgttctcctcccctcctgtgatcctcctcatttc
ctttctcatcttcctgatggtgggatgaaggaagcctccc-3′

N.B. This sequence is for the Ha PrP protein alone and does not include 5′ and 3′ terminal restriction sites necessary for cloning into the pRSET plasmid.

1. Excise the Ha PrP gene from the plasmid using appropriate restriction enzymes.

Follow the instructions that come with the restriction enzymes to determine the amount of enzyme, plasmid DNA and buffer to use.

2. Cut open the pRSET plasmid using the same restriction enzymes as those used to excise the PrP gene from the plasmid.

Follow the instructions that come with the restriction enzymes to determine the amount of enzyme, plasmid DNA and buffer to use.

3. Gel purify both the linear plasmid and the PrP gene.

- Prepare a 1% w/v agarose gel.
- Add DNA loading dye to the DNA samples.
- Load the molecular weight marker, the cut plasmid DNA and the excised PrP gene into separate wells on the agarose gel.
- Set voltage and time parameters on the electrophoresis equipment in accordance with manufacturer's instructions for a DNA gel.
- Run the agarose gel until the DNA loading dye approaches the bottom of the agarose gel.

N.B. The gene insert (approx. 800 bp) and linear plasmid (approx. 3000–4000 bp) may be identified by their molecular weight. The molecular weight lane marker should be used as a reference for this. Both lanes may contain one or more unexpected bands due to incomplete digestion of the plasmid DNA. Bands at the incorrect molecular weight should not be used.

4. Excise the bands containing the gene insert and the linear plasmid.

- Lay the gel on a UV-light box.
- Wearing appropriate personal protective equipment – including a UV eye shield – take a scalpel and excise the bands of interest from the gel.
- Deposit each band's gel piece(s) into an Eppendorf.

N.B. Gel pieces can be frozen at -20 °C and stored for up to two weeks as required.

5. Purify the DNA obtained from the bands.

Using a commercial DNA-gel extraction kit, and in accordance with the kit instructions, isolate the DNA obtained in each of the excised bands by spin-purification.

As an alternative to a commercial DNA-gel extraction kit, there are protocols available online or in the textbook 'Molecular Cloning – A Laboratory Manual' by Sambrook and Russell [5].

6. Quantitate the amount and purity of the DNA obtained.

- Measure the absorbance of the DNA solution at 260, 280 and 320 nm.
- Calculate the amount and purity of DNA obtained using the equation below.

$$\text{dsDNA concentration } (\mu g/ml) = (Abs_{260} - Abs_{320}) \text{ x (dilution factor) x } (50\ \mu g/ml/cm)$$

$$\text{Mass of DNA } (\mu g) = \text{Concentration } (\mu g/ml) \text{ x ml solution}$$

$$\text{Purity} = \frac{(\text{Abs } 260 \text{ nm})}{(\text{Abs } 280 \text{ nm})}$$

N.B. The DNA is of suitable quality for further use if it has a purity ratio in the range 1.7–2.0. As a desirable yield, the ligation in the next step requires around 75 ng of double stranded insert and 25 ng of linear insert.

If the purity and amount of DNA obtained from the previous purification step is inadequate, remedial 'trouble-shooting' action should be attempted before progressing to the next steps. This action is usually detailed in the manual accompanying the DNA extraction kit.

7. Ligate the gene into the plasmid.

In this step the Ha PrP gene is ligated into the new plasmid. A 1:3 ratio of plasmid to insert is desirable. Follow the instructions provided by the manufacturer of the ligase used.

N.B. Best practice is to store some of the plasmid obtained as a stock for transformation, should the glycerol bacterial stock (step 10) fail.

8. Transform the ligation reaction product into the BL21 DE3 Rosetta *E. coli*-competent cells.

Follow the instructions provided by Novagen. Alternatively, there are protocols available online or in the textbook 'Molecular Cloning – A Laboratory Manual' by Sambrook and Russell [5].

9. Isolate a pure bacterial colony.

- Inoculate 2-5 ml of LB medium with a single stab from the bacterial colony obtained in the previous step.
- Grow this bacterial culture up overnight at 30°C and 180 rpm in the presence of an appropriate quantity of antibiotic to which the plasmid has resistance.
- Inoculate 500 µL of various dilutions of the overnight culture onto agar plates which contain an appropriate antibiotic.
- Grow these colonies in a stationary incubator overnight at 30°C.

10. Prepare a glycerol stock for future cultures.

- Choosing an agar plate which has discrete and well-defined colonies, pick four colonies and use them to prepare four overnight starter cultures.
- Prepare four 50% v/v solutions of sterile glycerol in sterile water (e.g. 4 x 500 µL).
- Add an equal volume of the overnight culture to these glycerol solutions, mixing well. The relative final glycerol concentration is 25%.
- Store the glycerol stock at -80°C.

N.B. Glycerol stocks are stable for several months or even years, depending on the host bacterium, and provide an easy way to inoculate future cultures during recPrP production runs.

Bacterial culture

1. Prepare a 'starter' culture.

- Using a sterile toothpick or pipette tip, dip the narrow end into the glycerol stock and use it to inoculate 2–15 ml of antibiotic impregnated TB medium.
- Grow overnight at 30°C and 180 rpm.

2. Inoculate the 'grow-up' cultures.

Use 1–3 ml of starter culture to inoculate four 500 ml Overnight Express Instant TB Medium (OETB) cultures.

- Add appropriate antibiotic(s) to the cultures.
- Add glycerol (final concentration = 10 ml/L) to the cultures.
- Grow the cultures at 30°C, 180 rpm in non-baffled 2.5 L sterile plastic flasks.

N.B. Around 80–150 mg recPrP is expected from 2L of 'grow-up' culture. The OETB media mix does not require manual isopropyl β-D-1-thiogalactopyranoside (IPTG) induction by the operator.

3. Pelleting of bacterial cells.

- Once the OETB culture is complete, pellet the bacterial cells at 3000 x RCF for 15 minutes.
- Discard the supernatant, retaining the cell pellets in the Falcon tubes.
- The Falcon tubes are convenient storage containers for subsequent lysis steps.
- Cell pellets may be stored at this stage for up to six months at -80°C.

N.B. Expected pellet yield is 9 g/L culture under the specified growth conditions. Pellets should be orange or custard-yellow in colour. Expected yield is around 2g of cell pellet per Falcon tube when the freezing step has been reached.

Protein purification

1. Buffer preparation.

- Prepare the buffers as described in the annex. The pH tolerances are +/- 0.05 units.
- Filter the buffers through a 0.22 µ filter and de-gas prior to use.

N.B. The purification can be performed at 18–20°C without detriment to the protein; however the dialysis step should be at 0–4°C. The water used to dilute the dialysis buffer does not have to be de-gassed.

2. Double freeze-thawing of cells.

- Remove the pellets from the -80°C freezer and allow them to warm up for 15 minutes at room temperature.
- Return them to the -80°C freezer for 15 minutes.
- Remove the pellets from the -80°C freezer and allow them to warm up for 15 minutes at room temperature.

3. Isolate the inclusion bodies.

- Re-suspend the pellets in Bug Buster Master Mix (15 ml/2g of cell pellet).
- Place the suspended pellets on a benchtop rocker for 30 minutes at 10 rpm.
- Pellet IBs (and cell debris) by spinning at 13 500 x RCF for ten minutes at 4°C.

N.B. Do not vortex during this step. This has been reported anecdotally to adversely affect the final recPrP yield.

4. First wash of the inclusion bodies.

- Re-suspend the IBs and debris in the same volume of Bug Buster Master Mix as was used in the previous step.
- Place the suspended IBs and debris mix on a benchtop rocker for ten minutes at low rpm.
- Pellet IBs (and cell debris) by spinning at 13 500 x RCF for ten minutes at 4°C.

5. Second wash of the inclusion bodies.

- Using **one tenth** of the volume of Bug Buster Master Mix used in the previous step, dilute the Bug Buster Master Mix with dd.H_2O to obtain a 10% Bug Buster solution.
- Re-suspend the IBs and debris in this solution.
- Place the suspended IBs and debris mix on a benchtop rocker for ten minutes at 10 rpm.
- Pellet IBs (and cell debris) by spinning at 13 500 x RCF for ten minutes at 4°C.

N.B. At this point in the protocol, the washed inclusion bodies can be frozen at -80 °C and stored. After isolation and washing, the inclusion bodies should be milky-white. When frozen, they should be a dark yellow-green in colour.

6. Buffer equilibration of the Affinity Matrix.

The equilibration is comprised of three buffer washes.

- Add 17 ml of the Nickel NTA Superflow slurry to each of the 6 x 50 ml Falcon tubes.
- Wash the resin in each tube with 10 ml of the 6 M Guanidine denaturing buffer ('6DB') using a rocker at 10 rpm for five minutes.
- Spin the 1500 RCF for five minutes to sediment the matrix.
- Wash the resin in each tube with 10 ml of the 6 M Guanidine denaturing buffer ('6DB') using a rocker at 10 rpm for five minutes.
- Spin the 1500 RCF for five minutes to sediment the matrix.
- Wash the resin in each tube with 10 ml of the 6 M Guanidine denaturing buffer ('6DB') using a rocker at 10 rpm for five minutes.
- Spin the 1500 RCF for five minutes to sediment the matrix.
- Decant off the supernatant and replace with enough '6DB' to afford 20 ml slurry volume per Falcon tube.

7. Lyse inclusion bodies.

- Re-suspend each inclusion body pellet in 15 ml of the 8M denaturing buffer ('8DB').
- Pellet the debris for ten minutes at 4°C and 13 500 RCF.
- Add the supernatants (6 x approx. 15 ml) to the equilibrated matrix slurries in the above step.
- Re-suspend the debris in a further 15 ml of the 8M denaturing buffer ('8DB').
- Pellet the debris for ten minutes at 4°C and 13 500 RCF.
- Add the supernatants (6 x approx. 15 ml) to the equilibrated matrix slurries in the above step.

N.B. Remember to pre-chill the 1 x dialysis buffer in preparation for the dialysis step.

8. Load lysate onto the nickel NTA resin.

- Mix the inclusion body lysates with the equilibrated NTA resin (in six Falcon tubes).
- Shake on a rocker at 10 rpm for 60 minutes at 18–20°C.
- Spin down the matrix at 1 500 RCF for five minutes at 18-20°C and discard the supernatant.

9. Purge the FPLC system.

Equilibrate the FPLC system with 6M Denaturing Buffer '6DB' (Line A) and refolding buffer 'RB' (Line B).

10. Pack the column with the nickel NTA resin.

- Pour the equilibrated NTA resin slurry into a suitable column and bed the matrix.
- Connect the column to the FPLC.

11. Purify recPrP – wash non-PrP protein off the column *(25 minutes).*

Pump isocratic 6M denaturing buffer '6DB' for five minutes at 5 ml/min (approx. 0.25 column volumes) using line A.

12. Purify PrP – refold recPrP on the column *(3 hrs 35 minutes).*

- Run a gradient at 5 ml/min for 200 minutes (approx. 10 column volumes), switching from the current 6M denaturing buffer '6DB' (line A) to the renaturing buffer 'RB' (line B).
- Once the gradient is complete, continue pumping isocratic renaturing buffer 'RB' (line B) over the column for a further 15 minutes (approx. 0.75 column volumes).

13. Purify PrP - Elute recPrP *(1 hr 20 minutes).*

- Once the refolding step is complete, remove line A from the 6M denaturing buffer '6DB' buffer and put it into the Elution Buffer 'EB'.

N.B. Remember to purge pump A and line A of the former '6DB' buffer before starting the elution.

- Run a gradient at 5 ml/min for 60 minutes (approx. three column volumes), switching from the current renaturing buffer 'RB' (line B) to the elution buffer 'EB' (line A). Collect this volume in fractions.

N.B. The recPrP peak typically begins to elute when the buffer gradient is comprised of 50% elution buffer. The peak is broad (approx. 30 minutes) with little to no shoulder. Collect the middle 75% of the peak fraction-wise and directly into 1 x dialysis buffer at a ratio of one volume elute to two volumes of buffer. This increases final recPrP yield by reducing loss to spontaneous fibrillisation.

Only once the elution has been shown to be reproducible on the instrument should the elute be collected as one volume into a beaker containing 1 x dialysis buffer.

- Once the gradient is complete, continue pumping the isocratic elution buffer at 5 ml/min over the column for a further 20 minutes (approx. one column volume). Collect this volume in fractions.

N.B. The Abs_{280nm} trace will rise slowly during the gradient since imidazole absorbs at 280 nm. The elution endpoint comes at the conclusion of this isocratic step. The earliest and latest eluting fractions are unsuitable for use in RT-QuIC.

14. Combine desired fractions and dialyse overnight.

- By referring to the UV trace on the FPLC, calculate which of the fractions contain the middle 75% of the elution.
- Combine these and filter them through a 0.22 µ filter.
- Dialyse the diluted and filtered fractions overnight against pre-chilled 1 x dialysis buffer 'DB' at a ratio of 0.45L fractions to 10L dialysis buffer (approx. 20 x diluted fraction volume).

N.B. Filtration of the recPrP solution PRIOR to dialysis is critical to preventing premature fibrillisation and a substantial yield loss.

Prepare the same volume of fresh 1 x dialysis buffer so that it is pre-chilled for the next day's buffer exchange.

15. Exchange buffer.

Exchange the dialysis buffer for fresh, pre-chilled 1 x dialysis buffer and continue dialysis for five hours.

16. Determine recPrP concentration.

Determine the dialysed concentration of the recPrP – for example, using Abs_{280nm}.

ε_{280nm} = 62005 $M^{-1}cm^{-1}$ (1 g/L = 2.702)
MW = 23.0603 kDa.

17. Concentrate recPrP.

Using spin concentrators, concentrate the recPrP to give a final concentration of between 0.3 and 0.5 mg/ml.

18. Quality control.

- Determine the purity of the concentrated recPrP, for example, using SDS-PAGE and western blot.
- If substantially contaminated, do not proceed to the next step.
- RecPrP quality is determined by testing in RT-QuIC against the control brain homogenates and known positive and negative CSF samples. The recPrP batch passes if it produces the expected signal responses, as set out in the data analysis section.

N.B. A 'double overlapping band' for recPrP is often observed when purifying with this method but it has no detrimental effect on the RT-QuIC assay. However, only very small amounts of other bands should be accepted.

If the recPrP is substantially contaminated, a lesser yield of functional recPrP can be salvaged by performing an additional purification step, such as ion-exchange chromatography.

19. Washing the resin.

- Switch both lines to denaturing elution buffer ('DEB').
- Pump the denaturing elution buffer at 5 ml/min over the column for 30 minutes (approx. 1.5 column volumes).
- Switch both lines to water.
- Pump the water at 5 ml/min over the column for 30 minutes (approx. 1.5 column volumes).

20. Re-condition the resin.

- Transfer the resin from the column into six 50 ml Falcon tubes (approx. 17 ml resin/tube).
- Add demineralised water (15 ml) to each tube and spin the resin down at 3 000 RCF for five minutes.
- Wash 1 - Decant off the supernatant and wash with **5 M NaOH** (15 ml).
- Spin the resin down at 3 000 RCF for five minutes.
- Wash 2 - Decant off the supernatant and wash with **5 M NaOH** (15 ml).
- Spin the resin down at 3 000 RCF for five minutes.
- Wash 3 - Decant off the supernatant and wash with **5 M NaOH** (15 ml).
- Spin the resin down at 3 000 RCF for five minutes.

N.B. A colour change of blue to brown may be observed during this step.

- Wash 4 - Decant off the supernatant and wash with **demineralised water** (15 ml).
- Spin the resin down at 3 000 RCF for five minutes.
- Wash 5 - Decant off the supernatant and wash with **demineralised water** (15 ml).
- Spin the resin down at 3 000 RCF for five minutes.
- Wash 6 - Decant off the supernatant and wash with **renaturing buffer** ('RB', 15 ml).
- Spin the resin down at 3 000 RCF for five minutes.
- Wash 7 - Decant off the supernatant and wash with **renaturing buffer** ('RB', 15 ml).
- Spin the resin down at 3 000 RCF for five minutes.

N.B. If storing for 1–2 weeks, store the matrix in renaturing buffer at 4°C. If storing for longer, use a 20% ethanol/water solution at 4°C.

Performing the RT-QuIC assay

Safe handling and disposal of sCJD brain homogenates

All work involving the handling of sCJD brain homogenates should be carried out in a Class 2 MBSC in a Category 3 laboratory. This specifically includes, but is not limited to, the seed dilutions, loading of well plates and the disposal of sCJD contaminated materials after the experiment is complete. Disposable pipette tips can be sealed inside a Sharpsafe and kept within the MBSC.

Eppendorfs and well plates can be disposed of by soaking in 2M NaOH solution at room temperature for a minimum of one hour in an alkali impervious container with an airtight seal.

Preparing the brain homogenate controls

- Prepare N2 buffer, as detailed in Annex 1.
- Collect frontal cortex samples from suitable brains. Around 100 mg each of sudden-death, Alzheimer's diseased and sCJD brains are required.
- Prepare 10% w/v solutions of these brain samples in N2 buffer, along with a reference sample containing a known concentration of recombinant Hu PrP.
- Collect a 100 µL aliquot of the sCJD sample solution and using Proteinase K (PK, 2 µL, 2 mg/ml), digest it at 37°C for one hour. The other controls are not treated with PK.
- Prepare between 10–12 serial 1:10 dilutions of the PK-digested sCJD brain sample.
- Run a western blot using these dilutions against a known concentration of Hu PrP. Visualise bands using anti-prion protein antibody 3F4 epitope, in accordance with the manufacturer's instructions.
- Compare the combined band densities of each digested sCJD dilution (three bands) to that of the known Hu PrP concentration (one band) and calculate stock concentration.
- Dilute the sCJD stock in N2 buffer as required to afford a Hu PrP concentration of 100 fg /2 µL.
- The brain homogenate controls should be aliquoted out and stored at -80°C until required.

Preparing the master mixes

One master mix is prepared for the brain homogenate controls and one master mix is prepared for each group of CSF volumes being tested (i.e. one master mix for all the samples to which 30 µL of CSF will be added, one master mix to which 15 µL of CSF will be added and one master mix to which 7 µL will be added.)

CSFs are always tested at 30 µL volumes in the first instance and are only tested at 15 µL and 7 µL volumes if the results of the 30 µL tests are equivocal.

- Master mix for the brain homogenate control samples – Calculate enough master mix to make 17 wells (16 control wells + 1 extra).
- Master mix for the CSF test samples – Calculate the total number of CSF samples to be analysed at each CSF volume as follows:

  Total number of wells for the master mix calculation = (number of CSF samples at X µL x 4) + 1.

For each master mix, multiply each component in the table below by the total number of wells in that master mix to give each master mix's composition.

N.B. The Ha recPrP must be spin-filtered before being added to the master mix. The concentration of recPrP must be determined in order to calculate the correct volume of water and recPrP to add to each master mix. This process is detailed in the next step.

Table 7. Master mix in a single well

Reagent	Volume per well
PBS, 5x conc	20 µL
Sodium chloride solution, 2M	8.5 µL
EDTA, 100 mM	1 µL
1 x ThT, 1 mM (dilute from 10mM stock)	1 µL
Water, molecular biology grade	(100 - 30.5 - recPrP – Sample) µL
recPrP (add last)	Must be calculated – see next step.

Preparing and quantitating the recPrP

- Thaw the frozen recPrP solution at RT then spin filter at 3 000 x RCF and 4°C through 100 kDa MWCO spin-filtration units. Quantitate the recPrP solution concentration as described in the following steps. (Store the bulk filtered recPrP at 4°C until required.)
- Take two samples of the recPrP filtrate and dilute x 10 into a solution of 1 x PBS containing 0.1% SDS which has been passed through a 0.22 µ filter (e.g. 30 µL of recPrP filtrate diluted into 270 µL of 1 x PBS containing 0.1% SDS works well.)
- Measure the absorption of these samples at 280 nm. The results should agree within the limits of the instrument. Repeat the sampling process if they do not.
- Perform the following calculation to determine the volume of recPrP solution to be added to each well:

  $Abs_{280\ nm}$ x 10 = Neat $Abs_{280\ nm}$

  Neat $Abs_{280\ nm}$ ÷ 2.7 = neat conc (in mg/mL)

  10 µg/neat conc (in mg/mL) = volume in L to add per well (typically 20-40 µL).

  Volume to add per well x number of wells = volume of recPrP to add to each master mix.
- Combine enough recPrP with master mixes so that the final master mix compositions match that described in the table below. ThT is added as the penultimate step and the master mixes are vortexed immediately **before the recPrP is added**. The recPrP is always the last component to be added to the master mixes. Once the recPrP has been added, master mixes should be mixed **by gentle inversion x 3**, then subsequently handled with care to reduce accidental fibrillisation.

Table 8. Final master mix composition in a well

Reagent	Volume per well
Hamster Prion Protein	20–30 µL (10 µg after spin filtration)
PBS, 5x conc	20 µL
Sodium chloride solution, 2M	8.5 µL
EDTA, 100 mM	1 µL
ThT solution in water, 1 mM	1 µL
Water, molecular biology grade	(100 - 30.5 - recPrP – Sample) µL

N.B. The ThT is best handled in darkened conditions to minimise photobleaching, such as foil-wrapped Eppendorfs/tubes and in an unlit MBSC. As mentioned previously, the ThT should be made up at a 10 mM concentration (e.g. 32 mg ThT/10 ml water) to allow accurate weighing. The ThT should be solubilised at room temperature, then passed through a 0.22 µ filter. The yellow solution is stored in foil at 4°C until ready for use, at which point it is diluted ten-fold in water to afford a 1mM solution. This 10 mM solution should be freshly made for every RT-QuIC assay.

Preparing the RT-QuIC plate

- Load the RT-QuIC plate using the master mixes – i.e. Control Master Mix (98 µL) then add the control samples (2 µL) **or** Sample Master Mix (100 µL – CSF sample volume) then add the test CSFs (30 /15/7 µL).
- Once the 96-well plate has been filled with samples, seal the top of the plate using adhesive film and load it into the plate reader.

Table 9. The final composition of a well

Reagent	Concentration	Volume per well
Hamster Prion Protein	10 µg/100 µL	20–40 µL
PBS, 5x conc	50 mM PO_4 770 mM NaCl	20 µL
Sodium chloride solution	2 M	8.5 µL
EDTA	100 mM	1 µL
ThT solution in water	1 mM	1 µL
Water, molecular biology grade	-	(100 - 30.5 - recPrP – Water -Sample) µL
Samples	**Concentration**	**Volume per well (control or sample)**
Unseeded blank	1 x PBS	2 µL
Sudden-death brain homogenate	50 fg Hu PrP/µL[†]	2 µL
Alzheimer's diseased brain homogenate	50 fg Hu PrP/µL[†]	2 µL
sCJD brain homogenate	50 fg Hu PrPres/µL[†]	2 µL
CSF sample	Neat	30, 15 or 7 µL

[†]Concentrations are determined using western blot. The quantitation method is detailed above in the section 'Preparing the brain homogenate controls.'

N.B. The presence of blood in a CSF sample can suppress a positive response, increasing the likelihood of the sample returning a false negative. As a suggested limit, CSFs with red blood cell counts of ≤ 150 cell/mcL do not exhibit this effect.

RT-QuIC instrument protocol

Each 'measurement cycle' consists of two phases – shaking and reading. These phases differ in length depending on the instrument used. A batch script is used to call the shake and read scripts. Full details and examples of how these are written are listed in the appendix.

Briefly, the FLUOstar Optima and Omega phases are 14 minutes of shaking followed by one minute of reading the plate. The 14 minutes of shaking is programmed in as 7 x 2 minute shaking cycles on the instrument. The Optima and Omega take slightly different times to accelerate up to the specified shaking speed. This difference results in the Optima having a two-minute cycle consisting of 60 seconds of shaking and 60 seconds of rest, while the Omega has 86 seconds of shaking and 34 seconds of rest.

It can take up to 90 hours for a sample to give a clear positive result so the measurement cycles should be continued until the instrument has completed measurement cycle 361, at around 96 hours.

The RT-QuIC assay requires the FLUOstar OMEGA to shake at 900 rpm, which is above the maximum shake speed available through standard software options. However, the manufacturers can modify the software settings to allow this higher shake speed to be reached.

Annex 2 contains all required input parameters for instrument set-up.

It should be noted that the high sensitivity and specificity values reported for RT-QuIC were obtained using the recommended settings for the FLUOStar Optima instrument. The FLUOstar Omega has been tested at the NCJDRSU and delivered comparable results to the Optima. The use of the recommended conditions is supported by a large body of sample data and deviations from this protocol may lower the quoted sensitivity and specificity values for detection of sCJD.

Some laboratories may only have access to certain instruments and substrates or a limited access to CSF reference samples. A range of conditions for different instruments and substrates has been included in Annex 2.

Data analysis

Samples or controls are positive if **the two wells with the highest RFUs** exceed +3 standard deviations of the negative controls within the duration of the run, or at cycle 361. The number of positive wells out of each quadruplicate sample set is noted, as well as the time taken to reach the positive cut-off RFU value.

With regard to the brain homogenate controls, the unseeded, sudden-death and Alzheimer's disease controls should give a flat response. Only the sCJD controls should exhibit an increase in RFU.

Positive CSF samples frequently give between 2/4 and 4/4 positive results.

If only one well out of the four is positive, or if the fluorescent trace rises above the background but does not reach the cut-off value, this could either be:

- a negative CSF sample exhibiting a stochastic effect due to local well conditions – a 'false positive';
- a positive CSF sample where there are lots of PrP^{Sc} seeds in the wells. In this case, all the recPrP is templated onto many small plaques before the plaques can bind enough ThT to give a strong positive signal.

In cases where only one well out of the four is positive, the sample analysis should be repeated in a subsequent run using 15 µL CSF[1].

Table 10. Typical ranges for endpoint RFUs of two highest wells at Cycle 361

Sample type	N	Mean (RFU)	Standard deviation (RFU)
Unseeded (control)	16	16600	677
Sudden-death brain (control)	17	16749	566
Alzheimer's disease brain (control)	19	16709	753
sCJD disease brain (control)	21	146145	31264
30 CSF (positive sample)	57	92024	37972
30 CSF (negative sample)	126	16159	936

Values calculated using the recommended conditions for the FLUOStar Omega, as described in Annex 2.

[1] NCJDRSU has subsequently found that using 7ul of CSF is unreliable.

References

1. Wilham JM, Orrú CD, Bessen RA, Atarashi R, Sano K, Race B, et al. Rapid end-point quantitation of prion seeding activity with sensitivity comparable to bioassays. PLoS Pathog, 2010. 6(12):e1001217.
2. Peden AH, McGuire LI, Appleford NEJ, Mallinson G, Wilham JM, Orrú CD, et al. Sensitive and Specific Detection of Sporadic Creutzfeldt-Jakob Disease Brain Prion Protein Using Real-Time Quaking Induced Conversion. Prion, 2011. 5:75-75.
3. McGuire LI, Peden AH, Orrú CD, Wilham JM, Appleford NEJ, Mallinson G, et al. Real time quaking-induced conversion analysis of cerebrospinal fluid in sporadic Creutzfeldt–Jakob disease. Annals of Neurology, 2012. 72(2):278-285.
4. Atarashi R, Satoh K, Sano K, Fuse T, Yamaguchi N, Ishibashi D, et al., Ultrasensitive human prion detection in cerebrospinal fluid by real-time quaking-induced conversion. Nature Medicine, 2011. 17(2):175-178.
5. Sambrook JF, Russell DW. Molecular Cloning – A Laboratory Manual (Third Edition), Cold Spring Harbor Laboratory Press, 2001.

Annex 1.

Buffer recipes for recPrP purification

NB: the volumes given here are sufficient for ONE purification run under prescribed conditions. All buffers should be 0.22 µ filtered and (apart from the 20 x DIB) de-gassed before use.

'8DB' Denaturing buffer

38.5 g Guanidine hydrochloride

Final volume = 100 ml at pH 8.0

'6DB' Denaturing buffer

1.3 g $NaH_2PO_4.H_2O$
13.0 g Na_2HPO_4 (100mM total phosphate)
1.2 g Tris (10mM Tris)
580.0 g Guanidine hydrochloride (6.0 M)

Final volume = 1 L at pH 8.0

'RB' Refolding buffer

1.0 g $NaH_2PO_4.H_2O$
14.0 g Na_2HPO_4 (100mM total phosphate)
1.2 g Tris (10mM Tris)

Final volume = 1 L at pH 8.0

'EB' Elution buffer

2.3 g $NaH_2PO_4.H_2O$
1.2 g Na_2HPO_4 (100mM total phosphate)
0.3 g Tris (10mM Tris)
13.0g Imidazole hydrochloride (500 mM)

Final volume = 250 ml at pH 6.0

'DEB' Denaturing elution buffer

2.1 g $NaH_2PO_4.H_2O$ (100mM total phosphate)
4.4 g NaCl (500 mM NaCl)
87.0 g Guanidine hydrochloride (6M Gu.HCl)

Final volume = 150 ml at pH 4.0 (Denaturing elution buffer for cleaning Ni-NTA resin only)

20 x 'DIB' (20 x dialysis buffer)

24.0 g $NaH_2PO_4.H_2O$
4.0 g Na_2HPO_4 (200mM total phosphate)

Final volume = 1000 ml at pH 5.8

For dialysis dilute 20 x to give 10mM phosphate at about pH 6.5.

NB: This buffer does not have to be de-gassed before use. After diluting to 1 x, remember to pre-chill this buffer down to 0–4 °C in preparation for dialysis.

0.5 M NaOH solution

6.0 g NaOH

Final volume = 300 ml at pH 13

Recipe for N2 buffer

1 x PBS (10mM total phosphate)
0.1% SDS
1 x N2 supplement

Volume required = 10 ml at pH 7.4.

Annex 2.

Recommended and alternate assay parameters

	Recommended	Others have had success with:			Recommended	Others have had success with:		
Reader	OPTIMA	OPTIMA			OMEGA	OMEGA		TECAN
Recombinant PrP	Ham FL	Ham-Sh Chimeric	Ham FL	Hum FL	Ham FL	Ham FL		Hum FL
CSF volume[†]	30 or 15 µL	15 µL		5 µL	30 or 15 µL	20 µL	30 µL	5 µL
Shake conditions	600rpm 60s shake/60s rest	600rpm 60s shake/60s rest		750rpm 30s shake/30s rest	900rpm 86s shake/34s rest	900rpm 90s shake/30s rest		Max 30s shake/30s rest
Temperature	42°C	42°C		37°C	42°C	42°C		37°C
Criteria for positive result[‡]	Mean of two highest replicates out of 4 >10 000 RFU at 90 hrs	Mean of two highest replicates out of 4 >10 000 RFU at 80 hrs	Mean of two highest replicates out of 4 >6 000 RFU at 90 hrs	Mean of two highest replicates out of 4 >70% of baseline reading at 90 hrs	Mean of two highest replicates out of 4 >24 000 RFU at 90 hrs	Mean of two highest replicates out of 4 >20 000 RFU at 90 hrs	Average reading of all four wells >4 344 RFU at 90 hrs	At least two of six replicates >400 RFU at x hrs.

[†] *The CSF volume used in the assay is a speculative decision which balances the need for a robust and sensitive assay against the available amount of patient sample and the time taken to complete the measurement. The assay has a sensitivity of 91% and a specificity of 98% when 15 µL of CSF is used, as per the recommended FLUOstar Optima settings.[3]*

[‡] *The RFU 'cut-off' value used to determine whether an unknown sample is positive or negative is the mean end-point of a population of known negative samples + 3 standard deviations. The RFU value also varies with the 'gain' setting on the instrument, which is a control of the detector sensitivity. Two different research groups may therefore use the same instrument and reference samples but use a different RFU 'cut-off' limit.*

Batch script for running an RT-QuIC on either the FLUOstar Optima or Omega

```
Ask "Have you changed the experimental ID?" No:Halt Yes:

st1:='Date-Time-Operator'                          ;Experimental ID goes here

R_Temp 0.1                                         ;Switch on temperature monitoring

TargetTemp=42.0                                    ;set target temperature to 42oC

wait for temp >= TargetTemp                        ;Instrument waits until the target temperature has been reached

Ask "Reader is at 42oC. Insert plate and click Yes to proceed with test run" No:Halt Yes:R_PlateIn

d=400                                              ;sets loop for 400 times, every 15 minutes for 100 hours

for c:=1 to d do begin                             ;outer loop for multiple readings
    R_Run "RT-QuIC SHAKE"                          ;name of shake script

wait for 60s

    R_Run "RT-QuIC READ"                           ;name of read script

;merge horizontal(kinetic):
 Call "MergeReadings.exe <Datapath> <User>H ID1"

 End
```

Read script parameters, RT-QuIC FLUOstar Optima

Basic Parameters	
Microplate	Nunc 96
Optic	Bottom Optic
Number of multichromatics	1
Positioning delay	0.2 (Flying mode unchecked)
Filters	Excitation filter 450 and Emission filter 480
Gain	2000
Measurement start time	0
Number of flashed per well	20
Orbital averaging	(Unchecked)
Pause before plate reading	(Unchecked)

Layout	
Groups	(Unchecked)
Start value	1, Increase
Replicates	1, Horizontal
Reading direction	Snaking

Concentrations/volumes/shaking	
Standard concentration	Factor (greyed out)
Start volume	0
Volume	Factor, 1
Shaking options	Double orbital, shaking width 4mm, 150 rpm
Additional shaking	No shaking

Read script parameters, RT-QuIC FLUOstar Omega

Basic parameters	
Microplate	Nunc 96
Optic	Bottom Optic
No. of Multichromatics	1
Positioning delay	0.2 (Flying mode unchecked)
Filters	Excitation filter 450 and Emission filter 480
Gain	2000
Measurement start time	0
No. of flashed per well	20
Orbital averaging	(Unchecked)
Pause before plate reading	(Unchecked)

Layout	
Groups	(Unchecked)
Start value	1, Increase
Replicates	1, Horizontal
Reading direction	Snaking
Concentrations/volumes/shaking	
Standard concentration	Factor (greyed out)
Start volume	0
Volume	Factor, 1
Shaking options	Double orbital, 900 rpm
Additional shaking	No shaking

NB: Shaking at 900 rpm requires software update from BMG Labtech.

Shake script parameters, RT-QuIC FLUOstar Optima

Basic parameters	
Microplate	Nunc 96
Optic	Top Optic
No. of Multichromatics	1
Positioning delay	0.0 (Flying mode unchecked)
No. of kinetic windows	1
Filters	Excitation 450 and Emission filter 480
Gain	2000
Kinetic window 1	
No. of cycles	7
Measurement start time	0
No. of flashed per well and cycle	0
Cycle time	120
Basic parameters continued	
Orbital averaging	(Unchecked)
Minimum cycle time 1	(Greyed out)
Pause before plate reading	0

Layout	
Content	Sample
Groups	(Unchecked)
Start value	1, Increase
Replicates	1, Horizontal
Reading direction	Snaking

Concentrations/volumes/shaking	
Standard concentration	Factor (greyed out)
Start volume	0
Volume	Factor, 1
Shaking options	Double orbital, shaking width 1mm, 600 rpm
Additional shaking	Before each cycle
Shaking time	57s

Shake script parameters, RT-QuIC FLUOstar Omega

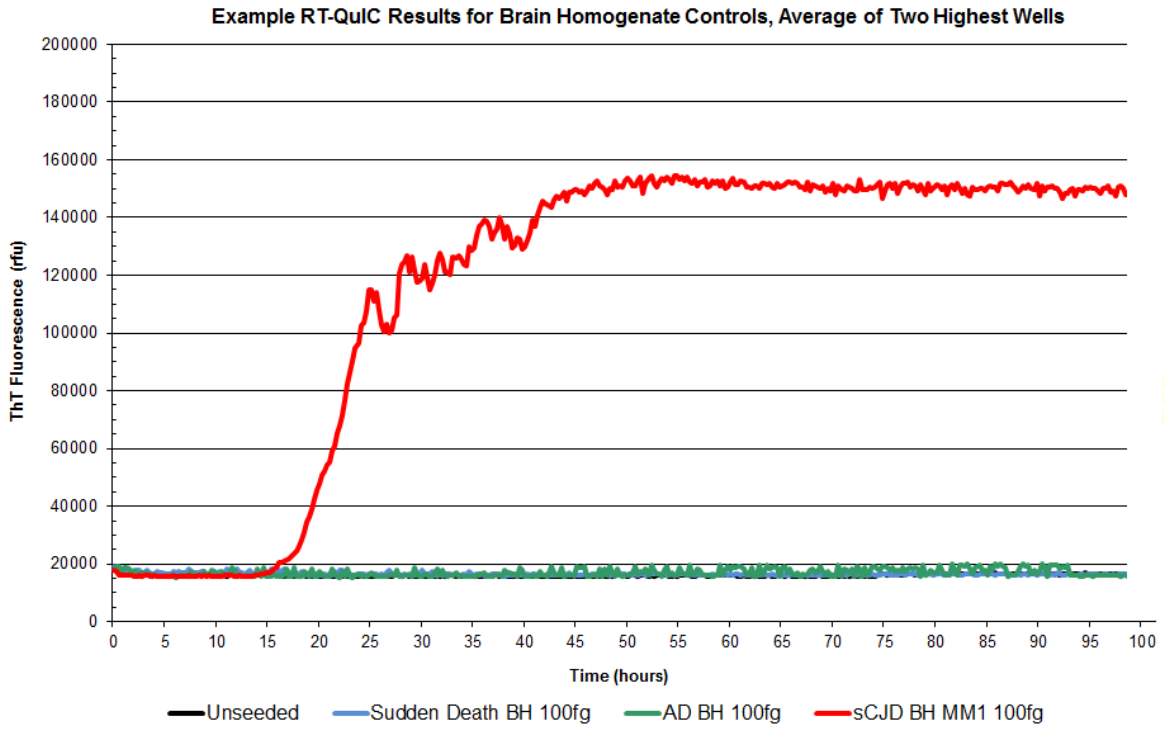
Example plate layout

When performing RT-QuIC on a 96 well plate with 4 control sets, there is space for 20 CSF samples.

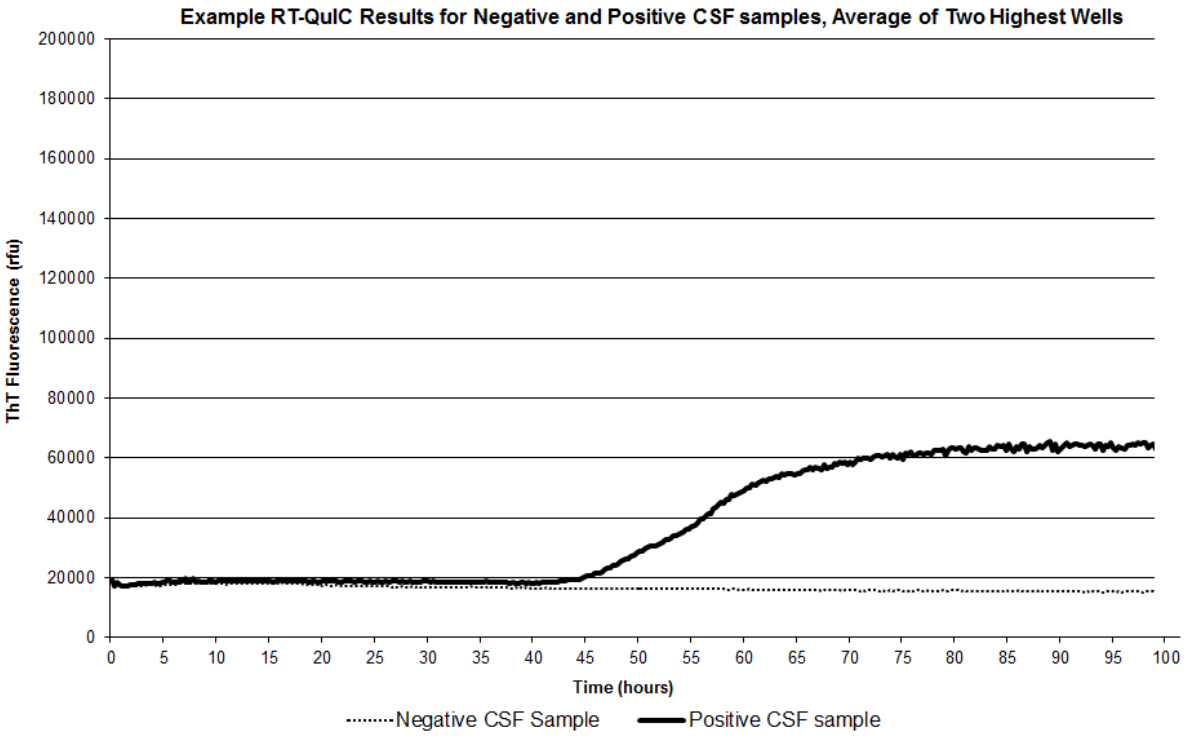
	1	2	3	4	5	6	7	8	9	10	11	12
A	**CONTROL 1** Unseeded Well	**CONTROL 1** Unseeded Well	**CONTROL 1** Unseeded Well	**CONTROL 1** Unseeded Well	**CONTROL 2** Sudden-death BH	**CONTROL 2** Sudden-death BH	**CONTROL 2** Sudden-death BH	**CONTROL 2** Sudden-death BH	**CONTROL 3** Alzheimer's Disease BH	**CONTROL 3** Alzheimer's Disease BH	**CONTROL 3** Alzheimer's Disease BH	**CONTROL 3** Alzheimer's Disease BH
B	**CONTROL 4** sCJD BH	**CONTROL 4** sCJD BH	**CONTROL 4** sCJD BH	**CONTROL 4** sCJD BH	**CSF Sample 1** 30 µL CSF	**CSF Sample 1** 30 µL CSF	**CSF Sample 1** 30 µL CSF	**CSF Sample 1** 30 µL CSF	**CSF Sample 2** 30 µL CSF	**CSF Sample 2** 30 µL CSF	**CSF Sample 2** 30 µL CSF	**CSF Sample 2** 30 µL CSF
C	**CSF Sample 3** 30 µL CSF	**CSF Sample 3** 30 µL CSF	**CSF Sample 3** 30 µL CSF	**CSF Sample 3** 30 µL CSF	**CSF Sample 4** 30 µL CSF	**CSF Sample 4** 30 µL CSF	**CSF Sample 4** 30 µL CSF	**CSF Sample 4** 30 µL CSF	**CSF Sample 5** 30 µL CSF	**CSF Sample 5** 30 µL CSF	**CSF Sample 5** 30 µL CSF	**CSF Sample 5** 30 µL CSF
D	**CSF Sample 6** 30 µL CSF	**CSF Sample 6** 30 µL CSF	**CSF Sample 6** 30 µL CSF	**CSF Sample 6** 30 µL CSF	**CSF Sample 7** 30 µL CSF	**CSF Sample 7** 30 µL CSF	**CSF Sample 7** 30 µL CSF	**CSF Sample 7** 30 µL CSF	**CSF Sample 8** 30 µL CSF	**CSF Sample 8** 30 µL CSF	**CSF Sample 8** 30 µL CSF	**CSF Sample 8** 30 µL CSF
E	**CSF Sample 9** 30 µL CSF	**CSF Sample 9** 30 µL CSF	**CSF Sample 9** 30 µL CSF	**CSF Sample 9** 30 µL CSF	**CSF Sample 10** 30 µL CSF	**CSF Sample 10** 30 µL CSF	**CSF Sample 10** 30 µL CSF	**CSF Sample 10** 30 µL CSF	**CSF Sample 11** 30 µL CSF	**CSF Sample 11** 30 µL CSF	**CSF Sample 11** 30 µL CSF	**CSF Sample 11** 30 µL CSF
F	**CSF Sample 12** 30 µL CSF	**CSF Sample 12** 30 µL CSF	**CSF Sample 12** 30 µL CSF	**CSF Sample 12** 30 µL CSF	**CSF Sample 13** 30 µL CSF	**CSF Sample 13** 30 µL CSF	**CSF Sample 13** 30 µL CSF	**CSF Sample 13** 30 µL CSF	**CSF Sample 14** 30 µL CSF	**CSF Sample 14** 30 µL CSF	**CSF Sample 14** 30 µL CSF	**CSF Sample 14** 30 µL CSF
G	**CSF Sample 15** 30 µL CSF	**CSF Sample 15** 30 µL CSF	**CSF Sample 15** 30 µL CSF	**CSF Sample 15** 30 µL CSF	**CSF Sample 16** 30 µL CSF	**CSF Sample 16** 30 µL CSF	**CSF Sample 16** 30 µL CSF	**CSF Sample 16** 30 µL CSF	**CSF Sample 17** 30 µL CSF	**CSF Sample 17** 30 µL CSF	**CSF Sample 17** 30 µL CSF	**CSF Sample 17** 30 µL CSF
H	**CSF Sample 18** 30 µL CSF	**CSF Sample 18** 30 µL CSF	**CSF Sample 18** 30 µL CSF	**CSF Sample 18** 30 µL CSF	**CSF Sample 19** 30 µL CSF	**CSF Sample 19** 30 µL CSF	**CSF Sample 19** 30 µL CSF	**CSF Sample 19** 30 µL CSF	**CSF Sample 19** 15 µL CSF	**CSF Sample 19** 15 µL CSF	**CSF Sample 19** 15 µL CSF	**CSF Sample 19** 15 µL CSF

Annex 3.

Example - unseeded and brain homogenate controls



Example - negative and positive 30 µL CSF samples



European Centre for Disease Prevention and Control (ECDC)

Gustav III:s Boulevard 40, 16973 Solna, Sweden

Tel. +46 858601000
Fax +46 858601001
www.ecdc.europa.eu

An agency of the European Union
www.europa.eu

Subscribe to our publications
www.ecdc.europa.eu/en/publications

Contact us
publications@ecdc.europa.eu

Follow us on Twitter
@ECDC_EU

Like our Facebook page
www.facebook.com/ECDC.EU

ECDC is committed to ensuring the transparency and independence of its work

In accordance with the *Staff Regulations for Officials and Conditions of Employment of Other Servants of the European Union* and the *ECDC Independence Policy*, ECDC staff members shall not, in the performance of their duties, deal with matters in which they may, directly or indirectly, have a personal interest that could impair their independence. Declarations of interest must be received from any prospective contractor before a contract can be awarded.
www.ecdc.europa.eu/en/aboutus/transparency

Publications Office
of the European Union