Document Information

Document Number:	CRO.SOP.00294
Document Title:	Method SOP: Detection of Prion Protein by ELISA in Rat Cerebrospinal Fluid for Sponsor 244
Document Version:	3.0
Document Status:	Effective
Date:	2/24/2020 4:55:35 PM

Approval By

Signer	Date and Time	Task	Reason for Signing
Matthew Moore	2/24/2020 8:05:46 AM	Task Completed (Step1 Approval): Approved	Originator
Gregory Lake	2/24/2020 8:53:00 AM	Task Completed (Step1 Approval): Approved	Reviewer
John Dan	2/24/2020 10:10:18 AM	Task Completed (QA Approval): Approved	Document Control
Jiewu Liu	2/24/2020 4:55:32 PM	Task Completed (Principal Investigator Approval): Approved	Principal Investigator Approval

Table of Contents

1.0 PRINCIPLE

This is a sandwich ELISA method, consisting of a capture antibody against prion protein coated onto microtiter plates and a detection antibody conjugated to biotin. Streptavidin-HRP and TMB substrate produce a colorimetric readout that is quantitated in a plate reader.

2.0 SIGNIFICANCE

The method's intended use is to monitor changes in PrP levels following administration of treatment for prion disease.

3.0 SCOPE

The method applies to the measurement of PrP in rat CSF for Sponsor 244.

4.0 DEFINITIONS

Abbreviation	Name
4PL/5PL	4/5 Parameter Logistic Curve
Ab	Antibody
BSA	Bovine Serum Albumin
CHAPS	3-[(3-Cholamidopropyl)dimethylammonio]-1-propanesulfonate hydrate
CSF	Cerebrospinal fluid
CV	Coefficient of Variation
HRP	Horseradish Peroxidase
LLOQ	Lower Limit of Quantitation
OD	Optical Density
PBS	Phosphate Buffered Saline
PrP	Prion Protein
QC	Quality Control
RE	Relative Error
rPrP	Recombinant Prion Protein
RT	Ambient Room Temperature
SD	Standard Deviation
TMB	3,3',5,5'-Tetramethylbenzidine
ULOQ	Upper Limit of Quantitation

5.0 RESPONSIBILITIES

The Principal Investigator is responsible for the maintenance and review of this method.

All analysts performing this method must execute each step according to this document.

6.0 REFERENCED DOCUMENTS

a) CRO.SOP.00066 – Bioanalytical Sample Analysis Process and Deliverables
b) LAB.SOP.00125 – Quality Control of Data and Documents
c) CRO.SOP.00076 – Bioanalytical Method Validation Process and Deliverables
d) QAU.Policy & Procedure.00038 – Deviation Management
e) QAU.Policy & Procedure.00067 – Procedure for Investigation Out-of-Specification Results
f) CRO.SOP.00086 – Sponsor and Project Code Assignment
g) CRO.SOP.00188 – Assay Batch Record Procedure
h) LAB.SOP.00060 – Documenting Reagent Preparation and Use and Inventory of Critical Reagents
i) LAB.SOP.00085 – Equipment Management
j) LAB.SOP.00118 – Quality Control of Reagent Performance
k) LAB.SOP.00119 – Reporting Significant Figures and Rounding
l) LAB.SOP.00127 – Study Role and Personnel Designation
m) QAU.Policy & Procedure.00035 – Good Documentation Practices
n) QAU.Policy & Procedure.00041 – Personnel Training Program and Records
o) QAU.Policy & Procedure.00056 – Requirements and Management of Standard Operating Procedures, Templates, and Work Instructions
p) QAU.Policy & Procedure.00061 – Storage, Organization, and Archiving of Electronic Study Records
q) SAM.SOP.00001 – Receiving, Inspecting, Labeling, and Distributing Incoming Materials
r) SAM.SOP.00003 – Sample, Specimen, and Test/Control Article Management
s) SAM.SOP.00006 – Sample Chain of Custody

7.0 SAMPLE OR SPECIMEN REQUIREMENTS

The required specimen is rat CSF stored at -80ºC.

8.0 CRITICAL EQUIPMENT, SUPPLIES, REAGENTS, AND MATERIALS

8.1 Critical Equipment

Name	Manufacturer	Model #	CBI ID
SpectraMax Plate Reader	Molecular Devices, Inc.	SpectraMax Plus 384 SpectraMax M5e	CB-1301 CB-1303
SoftMax Pro GxP v5.4.4 software	Molecular Devices, Inc.	GxP v5.4.4 software	N/A

8.2 Critical Materials, Reagents, and Supplies

8.2.1 Critical reagents require qualification per LAB.SOP.00118 and must be tracked as outlined in LAB.SOP.00060.

8.2.2 Replacement or substitution of critical reagents, materials, and supplies may require method re-evaluation and partial validation per CRO.SOP.00076.

Name	Manufacturer	Lot/Catalog #
Anti PrP Ab EP1802Y (capture antibody)	Abcam	ab52604
Anti PrP Ab 8H4 (detection antibody)	Abcam	Ab61409
Biotin-8H4 Detection Antibody	CBI	To be assigned
Recombinant Rat Prion Protein	Broad Institute	Batch 50, a CBI lot number will be assigned

9.0 GENERAL EQUIPMENT, SUPPLIES, REAGENTS, AND MATERIALS

9.1 General Equipment

9.1.1 General equipment, supplies, reagents, and materials may be replaced by equivalent substitutes as necessary.

Name
Refrigerator (4°C)
Freezer (-20°C and -80°C)
Vortex / Mixer
Plate Washer
Pipettes,single-channel and multi-channel
Plate Shaker

9.2 General Materials, Reagents, and Supplies

9.2.1 General equipment, supplies, reagents, and materials may be replaced by equivalent substitutes as necessary.

Name	Manufacturer	Catalog #
TMB Solution	Cell Signaling Technology	7004L
Stop Solution	Cell Signaling Technology	7002L
Rat cerebrospinal fluid (CSF)	BioIVT	RAT00CSFPZN
CHAPS	Sigma	10810118001
Milli-Q Water	Millipore	NA
Streptavidin-HRP	Thermo Scientific	21130
Nunc 96 wells Flat Bottom Immunoplates, MaxiSorp	Thermo Fisher Scientific	439454
0.22 µm Vacuum Filter System	Corning	CLS431098
Bovine Serum Albumin	Sigma	10735078001
1X PBS	Fisher Scientific	BP243820
Tween 20	Fisher Scientific	BP337-100

10.0 REAGENT PREPARATION

Volumes may be changed as necessary for usage provided that correct final concentration is maintained. Additionally, final concentrations listed may be changed during or at the conclusion of validation based on the data that has been generated.

Note: PrP is prone to pre-analytical variability, due to polypropylene adsorption. In order to minimize pre-analytical variability, samples, recombinant standards and QCs should be frozen after spiking with 0.03% CHAPS. Upon thawing samples should be pipetted up and down to mix, not vortexed. Additionally, samples and QCs should be handled gently and with minimum perturbation and plastic exposure. Ensure that each sample to be analyzed and compared is subject to the same amount of perturbation and plastic exposure.

Note: Upon first thaw, all CSF for QCs or validation samples must be stabilized with the addition of 0.03% (final concentration) of CHAPS. Per 1 mL of CSF, add 10 µL of 3% CHAPS. Mix gently by pipetting or inversion, aliquot (minimum aliquot size 40 µL) and freeze at -80ºC.

Note: bring all reagents to room temperature prior to use.

10.1 1X Wash buffer: 1X PBS with 0.1% Tween

10.1.1 Dilute 10X PBS to 1X PBS in milli-Q water. Then, dilute Tween-20 to 0.1% in the prepared 1X PBS. Example: 100mL 10XPBS + 1mL Tween + 900mL milli-Q water. Store at RT for up to 2 months.

10.2 Assay Buffer: 1X PBS with 5% BSA and 0.05% Tween

10.2.1 Dilute required amount of BSA, 10X PBS, and Tween in a portion of the required milli-Q water. Mix thoroughly. Add water to the final volume. Example: 25g BSA + 50mL 10X PBS + 0.25mL Tween + ~400mL water.

Add water to final volume of 500mL. Filter through a 0.22µm vacuum filter. Store at 4°C for up to 1 month.

10.3 Capture Ab Solution (2 µg/mL EP1802Y)

10.3.1 On day of use, dilute capture Ab EP1802Y to 2 µg/mL in 1X PBS. Mix by vortexing and discard remaining volume after use.

10.4 Detection Ab Solution (1 µg/mL Biotin-Detection Ab)

10.4.1 On day of use, dilute biotin-detection Ab to 1 µg/mL in Assay Buffer. Mix by vortexing and discard remaining volume after use.

10.5 Streptavidin-HRP Solution (27,000-Fold)

10.5.1 On day of use, dilute streptavidin-HRP 27,000-fold in Assay Buffer. Mix by vortexing and discard remaining volume after use.

10.6 Standards

10.6.1 Prepare high standard (Std01) by diluting stock rPrP to 40 ng/mL in Assay Buffer.

10.6.2 Make 6 serial 1.8-fold dilutions by adding, for example, 60 µL rPrP solution to 48 µL assay buffer, to produce the concentrations 22.2, 12.3, 6.86, 3.81, 2.12, and 1.18 ng/mL (Std02-07).

10.6.3 The last standard (Std08) is neat assay buffer.

10.6.4 Make a standard curve fresh from a frozen, undiluted rPrP stock every time.

10.7 QC Samples

10.7.1 QC samples will be prepared by combining and/or diluting lots of rat CSF that has been spiked with CHAPS and aliquoted per Section 10.0 prior to the first freeze and shipping.

10.7.2 Quantitative determination will be done each time a new batch of QCs is required. This determination should be carried out with both the current and new QC batches on the same plate wherever possible. Per CRO.Validation.01091 findings, the current QCs are prepared at 20.7 ng/mL (HQC), 7.97 ng/mL (MQC), and 4.44 (ng/mL).

10.7.3 Future preparations of controls should target the initial QC concentrations if possible.

11.0 PROCEDURE

11.1 Coat Plate

11.1.1 Prepare enough Capture Ab solution to add 100 µL to each plate well. Seal the plate and store overnight at 4ºC.

11.2 Wash

11.2.1 Wash plate 3 times with 300 µL Wash Buffer per well.

11.3 Block

11.3.1 Block plate by adding 250 µL Assay Buffer per well. Seal and incubate on bench top for 1 to 3 hours.

11.4 Wash

11.4.1 Wash plate 3 times with 300 µL Wash Buffer per well.

11.5 Standards, QCs and Samples

11.5.1 Dilute standards, QCs, and samples 8-fold (MRD) in assay buffer and add 100 µL of each to the plate per plate map. Seal and incubate on bench top for 60-75 minutes.

11.6 Wash

11.6.1 Wash plate 3 times with 300 µL Wash Buffer per well.

11.7 Biotinylated Detection Antibody

11.7.1 Prepare enough detection Ab solution to add 100 µL to each plate well. Seal the plate and incubate on bench top for 60-75 minutes.

11.8 Wash

11.8.1 Wash plate 3 times with 300 µL Wash Buffer per well.

11.9 Streptavidin-HRP

11.9.1 Prepare enough Streptavidin-HRP solution to add 100 µL to each plate well. Seal and incubate on bench top for 20-30 minutes.

11.10 Wash

11.10.1 Wash plate 3 times with 300 µL Wash Buffer per well.

11.11 TMB

11.11.1 Add 100 µL per well of TMB to plate. Cover and incubate at room temperature on bench. Intermittently check the color development targeting to have Std01 reach ~0.8 OD when read at 605 nm. If Std01 does not reach this OD within 30 minutes, proceed to the next step.

11.12 Stop

11.12.1 Add 100 µL per well of Stop Solution to plate. Mix well by shaking for a few seconds in plate reader.

11.13 Read

11.13.1 Read in Plate Reader at wavelength 450 nm with the background measured at 630 nm subtracted.

12.0 CALCULATIONS/DATA ANALYSIS

12.1 Softmax Software Calculations

12.1.1 The Softmax software will calculate the mean, SD and %CV of the absorbance values and fit the standards to a 4PL (unweighted) curve.

12.1.2 The Softmax software will calculate the back-calculated concentrations of the standards, QCs and samples, and the %RE of the standards and QCs.

13.0 ACCEPTANCE AND REJECTION CRITERIA

Samples, standards, and controls that do not meet listed criteria may be included/excluded at the discretion of the PI. The listed criteria may be changed based on findings during or at the conclusion of validation.

13.1 Calibration Curve

13.1.1 The %CV of the OD values for the duplicates for each point of the standard curve must be ≤20%, except for the lowest nonzero standard, which must be ≤25%. At least six of the seven non-zero standards must meet these criteria.

13.1.2 The average back-calculated result for each point of the standard curve must be within 20% RE of the expected concentration, except for the LLOQ, which must return an average back-calculated result within 25% of the expected concentration. At least six of the seven non-zero standards must meet these criteria.

13.1.3 One standard may be masked if acceptance criteria are not met. If the highest or lowest standard is masked, the ULOQ and LLOQ will be adjusted accordingly.

13.2 Quality Control Samples

13.2.1 The %CV of the duplicates for each QC level must be ≤30%.

13.2.2 QC acceptance criteria is specific to each preparation. Upon completion of quantitative determination for future preparations, new acceptance ranges will be calculated. Per CRO.Validation.01091 findings, the acceptable range for each of the currently prepared QCs is the nominal concentration ± 3 SD. The exact range of acceptable back-calculated concentrations for the QCs is below:

ID	Nominal Conc. (ng/mL)	-3 SD	+3 SD
HQC (VS-H)	20.7	13.0	28.4
MQC (VS-M)	7.97	4.00	11.9
LQC (VS-L)	4.44	1.61	7.28

14.0 REPORTING OF RESULTS

14.1 Round-Off Procedure

14.1.1 Observed and calculated values are rounded off to the number of decimal places that agree with the rules of significant figures as stated in LAB.SOP.00119.

14.2 Decimal Places

14.2.1 OD will be reported with 3 decimal places. Percentages will be reported with 1 decimal place.

15.0 CORRECTIVE ACTION

A run fails and will be repeated if the Acceptance Criteria for Standards and QCs is not met. Samples that do not meet Acceptance Criteria will be re-tested, possibly at a different dilution, if indicated by initial result. Any deviations from this SOP will be reported to the PI.

16.0 DOCUMENT REVISION HISTORY

Version #	Revisions Made	Rationale
1.0	N/A	New document
2.0	Section 10.0: Standards may be vortexed	The standards are made using recombinant prion protein which has not shown the same vulnerability to perturbation or plastic adsorption as endogenous prion protein used in QCs and samples.
2.0	Section 10.6: Standard concentrations and dilution folds updated	Concentrations updated to account for MRD. Dilution fold updated to reflect what was actually performed during validation.
2.0	Section 10.7: QC information added	QC determination has concluded so the newly available information on the current QC batch has been added

Version #	Revisions Made	Rationale
2.0	Section 11.11: TMB target OD clarified	Initial wording on the correct target OD while pre-reading was unclear
2.0	Section 11.13: Read wavelengths clarified	Clarified which wavelength should be subtracted
2.0	Section 12.1.1: curve fitting model clarified	The fitting model was clarified based on assay validation results.
2.0	Section 13.2: QC acceptance criteria added	Updated per change to Section 10.7
2.0	Attachment A: Document # corrected in header	Document# was incorrect
2.0	Attachment A: P# in equipment list updated	Updated to match new ABR format recommendations of lab management
2.0	Attachment A: Standards preparation updated	Updated per change to Section 10.6
2.0	Attachment A: Step 16 updated	Updated per change to Section 11.11
2.0	Attachment B: QC acceptance criteria updated	Updated per change to Section 10.7
3.0	Section 13.2.1 The %CV of the duplicates for each QC level was updated.	Based on assay validation results, the variability of the assay is higher than expected. The allowed %CV has therefore been adjusted from "≤ 25%" to "≤ 30%" to reflect the more realistic assay performance.

17.0 ATTACHMENTS

Attachment A: Assay Batch Record

Attachment B: Tech Review Checklist

RUN ID: _____Date: _____ Entries made by unless noted otherwise:_____

Assay Batch Record Day 1

Reagent Name	Lot number	Expiration Date
PBS		
Capture Antibody EP1802Y conc. _____		
Nunc MaxiSorp plate		

Equipment	ID	Calibration Due Date
Pipette P____		
Pipette P____		
Pipette P____		
Pipette P____		
Multichannel Pipette		
4°C Refrigerator		
Timer		

1) Prepare capture Ab solution by diluting capture antibody EP1802Y to 2.0 µg/mL in PBS.

   _____ µL capture Ab + _____ µL PBS

2) Add 100 µL/well of capture Ab solution to Maxisorp plate. Seal and incubate overnight at 4ºC.

   Start Date: _____ Start Time: _____

Originator Signature: _____ Date: _____

RUN ID: _____Date: _____ Entries made by unless noted otherwise:_____

Assay Batch Record Day 2

Reagent Name	Lot number	Expiration Date
Assay Buffer		
Biotin-8H4 Detection Ab conc. _____		
Stock rPrP conc. _____		
PrP QCs in CSF plus 0.03% CHAPS		
Streptavidin-HRP		
TMB		
Stop Solution		
Wash Buffer		

Equipment	ID	Calibration Due Date
Pipette P____		
Pipette P____		
Pipette P____		
Pipette P____		
Multichannel Pipette		
Plate Washer		
SpectraMax Plus Plate Reader		
-80°C Freezer		
4°C Refrigerator		
Timer		

3) Wash plate 3 times with 300µL/well of wash buffer and tap dry.

   Stop Date: _____ Stop Time: _____

4) Block by adding 250 µL/well of assay buffer to plate. Seal and incubate at RT for 1-3 hr on benchtop.

   Start Time: _____ Stop Time: _____

5) Prepare fresh standards from an aliquot of stock rPrP. Aliquots of stock rPrP should always be single-use.

RUN ID: _____Date: _____ Entries made by unless noted otherwise:_____

ID	Spike Volume, µL	Spike ID	Assay Buffer Volume, µL	Final Conc., ng/mL
Int1	10	rPrP Stock	490	11,400
Int2	10	Int1	490	228
Std01	20	Int2	94	40
Std02	60	Std01	48	22.2
Std03	60	Std02	48	12.3
Std04	60	Std03	48	6.86
Std05	60	Std04	48	3.81
Std06	60	Std05	48	2.12
Std07	60	Std06	48	1.18
Std08	N/A	N/A	100	0

6) Dilute standards, QCs, and samples 8-fold (MRD) in assay buffer by adding 30 µL standard, QC, sample + 210 µL assay buffer.

7) Wash plate 3 times with 300 µL/well of wash buffer and tap dry.

8) Add 100 µL/well of diluted standards, QCs, and samples per plate map in duplicate. Seal and incubate at RT for 60-75 min. All sample dilutions should be recorded on plate map if applicable.

   Start Time: _____ Stop Time: _____

9) Wash plate 3 times with 300µL/well of wash buffer and tap dry.

10) Prepare detection Ab solution by diluting biotin-labeled 8H4 detection antibody to 1.0 µg/mL in Assay Buffer.

    _____ µL detection Ab + _____ µL assay buffer

11) Add 100 µL/well of detection Ab solution. Seal and incubate at RT for 60-75 min.

    Start Time: _____ Stop Time: _____

12) Wash plate 3 times with 300µL/well of wash buffer and tap dry.

13) Prepare streptavidin-HRP solution by diluting streptavidin-HRP 27,000-fold in Assay Buffer.

    Int1:_____ µL detection Ab + _____ µL assay buffer (Dilution Fold_____)

    Streptavidin-HRP solution:_____ µL Int1 + _____ µL assay buffer (Dilution Fold_____)

14) Add 100 µL/well of streptavidin-HRP solution. Seal and incubate at RT for 20-30 min.

RUN ID: _____Date: _____ Entries made by unless noted otherwise:_____

Start Time: _____ Stop Time: _____

15) Wash plate 3 times with 300µL/well of wash buffer and tap dry.

16) Add 100 µL/well of TMB. Cover and incubate at RT on benchtop until Std01 reaches ~ 0.8 OD when read at 605 nm. If this OD is not achieved within 30 minutes, continue to next step.

Start Time: _____ Stop Time: _____

17) Add 100 µL/well of stop solution. Mix well on plate reader briefly and read at 450 nm with the background at 630 nm subtracted.

Originator Signature: _____ Date: _____

RUN ID: _____Date: _____ Entries made by unless noted otherwise:_____

Tech Review Checklist

[ ] Chain of custody form completed and correct

[ ] Plate map matches sample Worklist

[ ] Effective ABR was used.

[ ] Run ID on ABR, plate map and results

[ ] Equipment within calibration date.

[ ] Reagents within expiration date.

[ ] All blanks filled or N/A'd

[ ] All timed steps within limits

[ ] Calculations are correct

[ ] Results sample ID match plate map

[ ] ABR, plate map, and results are signed or initialed by originator.

Data Analysis

Leave checkboxes empty for any criteria below that are not met. Notify lead scientist and/or PI as soon as possible. As stated in method SOP, data generated that does not meet the below acceptance criteria may still be used at the discretion of the PI.

ID	Criteria
Curve	[ ] ≤ 1 point masked
Std01-06	[ ] RE ± 20% [ ] CV≤ 20%
Std07	[ ] RE ± 25% [ ] CV≤ 25%
QCs	[ ] CV ≤ 25% [ ] HQC back-calculated conc. 13.0 – 28.4 ng/mL [ ] MQC back-calculated conc. 4.00 – 11.9 ng/mL [ ] LQC back-calculated conc. 1.61 – 7.28 ng/mL

[ ] Following Tech Review, initial and date as tech reviewer on plate map, raw data, and any other attachments that were reviewed.

Reviewer Signature: _____ Date: _____