

Document Information

| Document Number: | CRO.SOP.00294 | |
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| | Cerebrospinal Fluid for Sponsor 244 | |
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Approval By

| Signer | Date and Time | Task | Reason for Signing |
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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.

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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
- 1) 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 -80°C.

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 |

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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

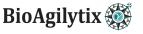
9.1.1 General equipment, supplies, reagents, and materials may be replaced by equivalent substitutes as necessary.

| 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.

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| 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 μ L of 3% CHAPS. Mix gently by pipetting or inversion, aliquot (minimum aliquot size 40 μ L) and freeze at -80°C.

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

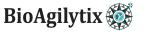
10.1 1X Wash buffer: 1X PBS with 0.1% Tween

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.

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Add water to final volume of 500mL. Filter through a $0.22\mu m$ vacuum filter. Store at $4^{\circ}C$ 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.

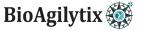
11.0 PROCEDURE

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 4°C.

11.2 Wash

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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 μ L per well of TMB to plate. Cover and incubate at room temperature on bench. Intermittently check the color development targeting to have Std01 reach \sim 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

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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.

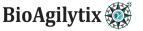
13.1 Calibration Curve

- 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 \leq 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:

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|---------------|---|--------------|
|---------------|---|--------------|

| 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 |

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| 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 "\le 25\%" to "\le 30\%" to reflect the more realistic assay performance. |

17.0 ATTACHMENTS

Attachment A: Assay Batch Record

Attachment B: Tech Review Checklist

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| CRO.SOP.00294 Attachment A Method S | | SOP: Detection of Prion Prion Prion Prion Prion Prion Prion Prior | • | Page 1 of |
|---------------------------------------|-------------------|---|-------------------------------|-------------|
| JN ID: | Date: | Entries made l | by unless noted otherwise: | |
| | | Assay Batch Record Day | 1 | |
| Re | eagent Name | Lot number | r Expiration | Date |
| | PBS | | | |
| Capture Antibo | dy EP1802Y conc. | | | |
| Nun | c MaxiSorp plate | | | |
| | | , | | |
| Equ | ipment | ID | Calibration Due I | Date |
| Pipett | e P | | | |
| | e P | | | |
| | e P | | | |
| Pipett | e P | | | |
| Multicha | nnel Pipette | | | |
| 4°C Re | frigerator | | | |
| T | imer | | | |
| | | n by diluting capture antibody E | P1802Y to 2.0 μg/mL in PB | S. |
| · | _ μL capture Ab + | μL PBS e Ab solution to Maxisorp plate. | . Seal and incubate overnight | at 4°C. |

Originator Signature: _____ Date: _____

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| 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 | | |
| Multichannel Pipette | | |
| Plate Washer | | |
| SpectraMax Plus Plate Reader | | |
| -80°C Freezer | | |
| 4°C Refrigerator | | |
| Timer | | |

| 3) Wash plate 3 times with $300\mu L/well$ of wash buffer and tap dry. | |
|--|---|
| | Stop Date: Stop Time: |
| 4) | Block by adding 250 $\mu L/\text{well}$ 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. |

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| 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 μ L standard, QC, sample + 210 μ L assay buffer.
- 7) Wash plate 3 times with 300 μ L/well of wash buffer and tap dry.

| 8) | • | d standards, QCs, and samples per plate map in duplicate. Seal and nin. 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 μ g/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.

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| RUN ID: _ | Date: | | Entries made by unless noted otherwise: | | | |
|--|---|------------|---|--|--|--|
| | Start Time: | Stop Time: | | | | |
| 15) | 15) Wash plate 3 times with $300\mu L/well$ of wash buffer and tap dry. | | | | | |
| 16) Add 100 μ L/well of TMB. Cover and incubate at RT on benchtop until Std01 reaches \sim 0.8 O 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 μ L/well of stop solution. Mix well on plate reader briefly and read at 450 nm with the background at 630 nm subtracted. | | | | | | |
| Origina | tor Signature: | | Date: | | | |

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| JN ID: | Date: | Entries made by unless noted otherwise: |
|----------------------|-----------------------------|--|
| | To | ech Review Checklist |
| [] Chain of custo | ody form completed and | |
| [] Plate map ma | tches sample Worklist | |
| [] Effective AB | R was used. | |
| [] Run ID on Al | BR, plate map and results | 5 |
| [] Equipment w | ithin calibration date. | |
| [] Reagents with | nin expiration date. | |
| [] All blanks fill | led or N/A'd | |
| [] All timed step | os within limits | |
| [] Calculations a | are correct | |
| [] Results samp | le ID match plate map | |
| [] ABR, plate m | nap, and results are signed | d or initialed by originator. |
| | | |
| Data Analysis | | |
| Leave checkboxes | s empty for any criteria b | elow that are not met. Notify lead scientist and/or PI as soon |
| as possible. As stat | ted in method SOP, data | generated that does not meet the below acceptance criteria |
| may still be used a | t 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-calcula | ted conc. 13.0 – 28.4 ng/mL |
| | [] MQC back-calcula | ted conc. 4.00 – 11.9 ng/mL |
| | [] LQC back-calculat | red conc. 1.61 – 7.28 ng/mL |
| | | |
| [] Following Too | oh Raviaw initial and a | late as tech reviewer on plate map, raw data, and any |
| | s that were reviewed. | and as teen reviewer on plate map, raw data, and any |
| Reviewer Signatur | | Date: |

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