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METHOD VALIDATION REPORT

DOCUMENT TITLE

Method Validation Report: Detection of Prion Protein by ELISA in Rat Cerebrospinal Fluid by Method CRO.SOP.00294 for Sponsor 244

DOCUMENT NUMBER

CRO.Validation.01091

CBI SPONSOR-PROJECT CODE

244A-002V

REGULATORY REQUIREMENTS

World Health Organization - Good Clinical Laboratory Practice (GCLP)

PRINCIPAL INVESTIGATOR

Jiewu Liu, PhD

INITIATION DATE

23JUL2019

COMPLETION DATE

Date this Report is approved by the Cambridge Biomedical Principal Investigator

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Template: LAB.Validation.00424, Version 3.0

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1.0 SUMMARY

The objective of this method validation was to evaluate the key performance parameters of CRO.SOP.00294 and assess the method's suitability for the intended purpose of measuring prion protein in rat cerebrospinal fluid by ELISA, in support of the clinical study for Sponsor 244.

The parameters evaluated in this validation were intra and inter-assay precision, upper and lower limit of quantitation determination, parallelism, and sample stability at room temperature, 4°C, and following multiple freeze thaw cycles.

The summary of the results in presented in Attachment A.

2.0 PRINCIPAL INVESTIGATOR COMPLIANCE STATEMENT

The Method Validation study titled "Method Validation Protocol: Detection of Prion Protein by ELISA in Rat Cerebrospinal Fluid for Sponsor 244", was conducted and reported in compliance with the Good Clinical Laboratory Practice Regulations set forth by the World Health Organization.

NOTE: Approval of this document by the Principal Investigator serves as the approval of this Compliance Statement.

3.0 QUALITY ASSURANCE STATEMENT

The Cambridge Biomedical Quality Assurance Unit has performed inspection on the method validation study "Method Validation Protocol: Detection of Prion Protein by ELISA in Rat Cerebrospinal Fluid for Sponsor 244", and findings were reported to Principal Investigator on the dates shown in the table below.

Date of Inspection	Phase Inspected	Document Number	Date Reported to Principal Investigator	Date Reported to Test Site Management
26SEP2019	In-Process	QAU.Reports.00409	26SEP2019	18Nov2019
12Dec2019	Report/Data	QAU.Reports.00457	13Dec2019	24Dec2019

NOTE: The Quality Assurance signature on this document serves only to document the inspections performed.

4.0 OBJECTIVES

The objective of this study was to validate method SOP CRO.SOP.00294, "Method SOP: Detection of Prion Protein by ELISA in Rat Cerebrospinal Fluid for Sponsor 244", for suitability for the intended purposes of a pharmacodynamic biomarker assay. This study evaluated intra and inter-assay precision, accuracy, parallelism, determination of limits of quantitation, and stability.

5.0 DEFINITIONS

Term	Definition
-80°C	Acceptable temperature range of -60°C to -90°C
CBI	Cambridge Biomedical Inc.
CHAPS	3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate hydrate
CSF	Cerebrospinal Fluid
CV	Coefficient of variation
HRP	Horseradish Peroxidase
LLOQ	Lower Limit of Quantification
MRD	Minimum Required Dilution
OD	Optical Density
QC	Quality Control (H, M, LQC are high, mid, and low QC, respectively). Prior to QC range determination these are the high, mid, and low Validation Samples
RE	Relative Error
VS	Validation Sample (VS-H, -M, -L are high, mid, and low, respectively)
SD	Standard Deviation
TMB	3,3',5,5'-Tetramethylbenzidine
ULOQ	Upper Limit of Quantification

Term	Definition
RT	Room Temperature, the acceptable range of 18°C to 25°C

6.0 REFERENCES

6.1 Study-Specific References

a. Method SOP - CRO.SOP.00294, "Method SOP: Detection of Prion Protein by ELISA in Rat Cerebrospinal Fluid for Sponsor 244"

b. LAB.SOP.00103 – Molecular Devices SpectraMax Plus 384 Microplate Reader Operation, Preventative Maintenance, and Calibration

c. LAB.SOP.00018 – Molecular Devices SpectraMax M5e Operation, Preventative Maintenance, and Calibration

6.2 General References

a. Guidance for Industry – Bioanalytical Method Validation, U.S. Department of Health and Human Services, Food and Drug Administration, Center for Drug Evaluation and Research (CDER), Center for Veterinary Medicine (CVM), May 2018

b. CRO.SOP.00076 – Bioanalytical Method Validation Process and Deliverables

c. SAM.SOP.00003 – Sample, Specimen, and Test/Control Article Management

d. SAM.SOP.00006 – Sample Chain of Custody

e. LAB.SOP.00045 – Technical Review of Data and Documents

f. LAB.SOP.00121 – Validation of Analytical Methods

g. LAB.SOP.00125 – Quality Control of Data and Documents

h. LAB.SOP.00127 – Study Role and Personnel Designation

i. QAU.Policy & Procedure.00017 – Archiving of Laboratory and Study Materials

j. QAU.Policy & Procedure.00031 – Amendment Procedure for Method Validation and Sample Analysis Documents

k. QAU.Policy & Procedure.00038 – Deviation Management

l. QAU.Policy & Procedure.00061 – Storage, Organization, and Archiving of Electronic Study Records

7.0 DEVIATIONS

7.1 Critical Deviations

None

7.2 Major Deviations

None

7.3 Minor Deviations

Deviation #	**1** **Unplanned**
Document #	CRO.Validation.01152
Title	Deviation Against LAB.SOP.00060 V5.0; Preparation of Biotin-8H4 Detection Ab Not Documented for 244A-002V
Description	Section 6.1.1 of LAB.SOP.00060 V5.0 states, "Laboratory personnel must complete Attachment A – Reagent Preparation Batch Record to document the preparation of reagents…" Analyst BR prepared Biotin-8H4 Detection Ab but did not record any part of its preparation. The identity of the reagent, the concentration, and the date that it was prepared were recorded directly on the vial.
Impact	It will be difficult to replicate the exact procedure followed by BR for biotin conjugation without any documentation. The lead scientist and PI could not determine whether BR performed a BCA test to get an exact concentration of the material or if he used the theoretical concentration.
Root Cause	Poor documentation by analyst
Corrective Action	MRM filled out as much of CRO.SOP.00241 Attachment A and B as possible and assigned a GLP number to the reagent. A BCA test was performed to verify the concentration of the conjugate but there was interference from a suspected additive in the diluent. The reagent will continue to be prepared in the assay using the concentration listed on the vial by BR. A deviation will be drafted. The assay will require re-optimization once biotin-8H4 reagent expires since the precise concentration of the current lot cannot be determined.

8.0 EXCEPTIONS TO PROTOCOL OR METHOD

Exception #	1 Planned
Description	Step 16 of CRO.SOP.00294 details colorimetric development of the assay plate. It states that the plate should incubate until Std01 reaches ~1.8OD. This statement does not provide sufficient information to properly read the plate. The full statement should be that: the plate should incubate until Std01 reaches ~0.8OD when read at 605 nm prior to stopping. When stopped and read at 450 nm, this will correspond with approximately ~1.8OD.
Impact	The method SOP and assay batch record will need to be updated but there was no impact on the integrity of the data.
Root Cause	Wavelength information for step 16 of assay batch record omitted while drafting method validation protocol.
Corrective Action	A memo (CRO.Validation.01092) with the full detail listed above was drafted on the same day that the error was discovered (24JUL2019 for Run01). The method SOP and assay batch record has been revised to clarify the wavelength information.

Exception #	2 Planned
Description	The standard preparation in the method CRO.SOP.00294 v1.0 (step 11.5.1 and Attachment A assay batch record (step 5) has a top concentration of 5 ng/mL and is diluted in 2.5-fold increments. Near the end of optimization by the previous lead scientist, it was decided that the bottom of this range was too close to background and, moving forward, would be diluted in 1.8-fold increments. Also, the previous lead scientist was not performing the MRD on the standard curve but failed to clarify that information.
Impact	The wider range and additional 8-fold dilution resulted in the lowest concentration being indistinguishable from background levels which led, in part, to Run01 failing to meet acceptance criteria.
Root Cause	Due to the sudden departure of the previous lead scientist, the current lead scientist was not aware of the decision to adjust the dynamic range until after the method SOP was made effective.

Exception #	2 Planned
Corrective Action	Corrections to the calculations and final concentrations for preparing the curve were footnoted on the assay batch record for each run. The method SOP and assay batch record have been updated.

Exception #	3 Planned
Description	The low validation sample demonstrated an inter-assay precision CV of 21.3% (see Table 3). This exceeds the acceptance criteria in the method validation protocol which is 20%. The initial criteria are an estimate of expected precision and there is an expectation that this estimate may need to be adjusted once validation data is available. All 6 values for the low validation sample inter-assay precision still fall within the newly established QC range for the LQC (see Table 4).
Impact	The method SOP will need to be updated. There is no impact to the data but loosening this acceptance criterion.
Root Cause	The observed %CV in the inter-assay precision data suggests that the amount of variability that should be expected for the assay is greater than 20%
Corrective Action	The acceptance criteria for QCs and samples have been updated in the method SOP.

Exception #	4 Planned
Description	The dilutions within the limits of quantitation in the parallelism experiment did not meet the %RE requirement listed in the method validation protocol (CRO.Validation.01065 v1.0, section 7.6). However, upon further research the PI found a suggestion in an industry white paper, “Points to Consider Document: Scientific and Regulatory Considerations for the Analytical Validation of Assays Used in the Qualification of Biomarkers in Biological Matrices.” This paper recommends that “The mean of the dilution-corrected

Exception #	4 Planned
	concentration range for all the dilutions that fall within the assay range must have a CV less than or equal to the CV set for the biomarker assay." Based on this new information, the CV criteria for the assay (20% for QCs and samples per the method SOP) will be raised to 30%. This wider criterion will now cover the range of CV's observed in all parallelism experiments (see Table 6).
Impact	Some sensitivity in the assay will be lost but, based on the validation data for both inter-assay precision and parallelism, this is the most realistic expectation for the assay's variability.
Root Cause	The assay has greater variability than initially estimated.
Corrective Action	This new criterion was applied to the parallelism in place of the %RE criteria listed in the method validation protocol. The acceptance criteria for QCs and samples have been updated in the method SOP.

Exception #	5 Unplanned
Description	In Run06, short-term stability was performed using validation samples due to a misinterpretation of the method validation protocol. The method validation protocol states that this assessment should be performed using "one preparation of pooled rat CSF." The validation samples are 3 different pools of rat CSF at three different concentrations of prion protein. Short-term stability was to be performed with a single pool to preserve reagent volume. The assessment in Run06 did not meet acceptance criteria for any of the 3 parameters and, although the data is presented in the report, only Run07's data will be used to establish short-term stability.
Impact	No impact on study as the exact specifications of the method validation protocol (CRO.Validation.01065 v1.0, section 7.7) were followed in Run07.
Root Cause	Method validation protocol wording was slightly unclear.

Exception #	5 Unplanned
Corrective Action	Performed short-term stability as intended with a single lot of rat CSF at a single concentration in Run07 for a shorter period of time at 4°C and RT and for fewer freeze/thaw cycles. Exact times are listed in Section 12.6.

Exception #	6 Unplanned
Description	In method validation protocol, CRO.Validation.01065, the acceptance criteria for all stability parameters does not specify how many replicates of each parameter must meet acceptance criteria individually to consider the parameter acceptable. In Run07, three replicates of a single level were analyzed for each short term stability parameter. Having at least 2 of 3 replicates meet acceptance criteria will be considered sufficient evidence that samples are stable at the condition for which they were tested.
Impact	Using this added criterion, the room temperature stability testing from Run07 changes from failing to passing.
Root Cause	Method validation protocol wording was unclear.
Corrective Action	No correction to the validation protocol will be made as there is no further scheduled stability testing.

9.0 PERSONNEL

Study Role	Name	Position Title
Test Site Management	Linda Robbie, PhD	Vice President & General Manager, Boston Operations
Principal Investigator	Jiewu Liu, PhD	Associate Director, Scientific Services
Lead Scientist	Matthew Moore	Scientist II

Study Role	Name	Position Title
Project Manager	Gregory Lake	Project Management Specialist
Analyst	Joseph Moy	Scientist I
Quality Control	Ryan Churchill	Quality Control Specialist
Quality Assurance	Mayada Guzman	QA Manager

10.0 MATERIALS AND EQUIPMENT

10.1 Reagents

Name	Manufacturer	Catalog#
Anti PrP Ab EP1802Y (capture antibody)	Abcam	ab52604
Anti-PrP Ab 8H4 (detection antibody)	Abcam	ab61409
Biotin-8H4 Detection Antibody	CBI	GLP030419P
Recombinant Rat Prion Protein	Broad Institute	PrP50

10.2 Critical Equipment

Description	Manufacturer	Model Number	CBI ID
SpectraMax Plate Reader	Molecular Devices, Inc.	SpectraMax Plus 384 SpectraMax M5e	CB-1301 CB-1303

10.3 Computerized Systems

Name	Developer	Software Version	Purpose
Orchard Harvest LIS	Orchard Harvest	11.0	Sample Management
SoftMax Pro GxP	Molecular Devices, Inc.	GxP v5.4.4	Analyzer controller and data analysis
Excel	Microsoft	Version 1902	Data Analysis

11.0 EXPERIMENTAL DESIGN

11.1 Method

CRO.SOP.00294: 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.

11.2 Sample Description and Processing

Sample Description: Rat cerebrospinal fluid

11.3 Statistical Methods

Equation 1: Calculation of Average Value

Average = (Sum of values / number of values)

Equation 2: Calculation of Standard Deviation (SD)

$$SD = \sqrt{\frac{Sum\ of\ (observed - average)^2}{n-1}}$$

Equation 3: Calculation of % Relative Error (%RE)

% RE = (Observed – Theoretical) / Theoretical x 100

Equation 4: Calculation of % Coefficient of Variation (CV)

% CV = (SD / Average) x 100

12.0 RESULTS

A summary of the runs performed in this validation can be found in Table 1.

12.1 Intra-Assay Precision

To determine the intra-assay precision of the assay, six duplicates of each validation sample were assayed in a single analytical run. The target for intra-assay precision was %CV ≤ 20%. These criteria were met with a maximum CV of 5.12%. See Table 2.

12.2 Inter-Assay Precision

To determine inter-assay precision, validation samples were assayed in seven independent analytical runs performed over 90 days by two different on operators on two different plate readers. The target for inter-assay precision was %CV ≤ 20%. The %CV criteria were met in the high and mid validation samples with a %CV of 12.4% and 16.6, respectively. The %CV of the low validation sample was calculated to be 21.3%. Acceptance criteria pertaining to %CV of QCs and samples will be raised accordingly as noted in Exception 3. See

Table 3.

12.3 Establishment of QC Range (Accuracy)

At the conclusion of the validation, the average back-calculated result for each validation sample was calculated. The acceptable range for each QC moving forward will be ± 3 standard deviations. The newly established ranges were retroactively applied to the inter-assay precision results. All data points fell within the acceptable range. See Table 4.

12.4 Establishment of Limits of Quantitation

To establish the limits of quantitation, the inter-assay %CV and %RE was calculated for each standard. The lowest and highest nominal standard concentrations that demonstrated %CV ≤ 25% and RE ≤ 25% were selected as the LLOQ and ULOQ, respectively. The LLOQ, 1.18 ng/mL (Std07), met the criteria with a %CV of 9.62% and %RE of 3.35%. The ULOQ, 40 ng/mL (Std01), met the criteria with a %CV of 0.27% and %RE of 0.181%. See

Table 5. This table also includes inter-assay CV calculated from mean OD at the request of the client.

12.5 Parallelism

To establish parallelism, CSF samples from 3 individual rats stabilized with 0.03% CHAPS were serially diluted in assay buffer in singlicate starting with 1:8 continuing to 1:256 in 2-fold steps. The measurements falling within the limits of quantitation were corrected for their dilution. The back-calculated concentration was compared to the least dilute data point by calculating the %RE. There was a preparation error in the parallelism test performed in Run05 that resulted in an additional 8-fold dilution of all parallelism samples. Additional parallelism tests were performed in Run06 and Run07. The initial criterion for parallelism was that all in-range back calculated results would have a %RE $\leq$ 25%. This was not met for any parallelism sample in Run06 or Run07. The parallelism was assessed with an alternative strategy by calculating the CV of all back-calculated concentrations within the limits of quantitation and comparing that to the CV acceptance criteria for QCs and controls in the assay (see Exception 4 for details). The observed CVs in Runs 05-07 were 26.2%, 16.9%, and 27.3%, respectively. The CVs are considered to be acceptable and in line with the inter-assay precision. See

Table 6.

12.6 Stability

To determine short-term stability at 4°C and RT, one preparation of pooled rat CSF with 0.03% CHAPS that had been aliquoted and stored at -80°C was subjected to the conditions described below and analyzed in the assay along with freshly thawed aliquots for reference. In order to meet acceptance criteria, the %RE of stability samples must be ≤ 20% when compared to the reference result. Initially, validation samples were used for this experiment. They were stored at 4°C and RT overnight for 20 hr 29 min and 20 hr 44 min, respectively. The stressed samples did not meet the %RE acceptance criteria at these times. The maximum %RE for 4°C was -43.6% and -59.3% for RT. Results suggested that the samples should be stressed for less time, so the experiment was repeated. The repeat of stability in Run07 used a single lot of rat CSF at one concentration, which is more in line with how the validation protocol should have been interpreted (see Exception 5). Three aliquots of the single rat CSF lot each were stored at 4°C and RT for 8 hr 37 min. All aliquots stored at 4°C met acceptance criteria with a maximum %RE of 6.7%. 2 of 3 aliquots stored at RT met acceptance criteria with a maximum passing %RE of -6.7%. The third aliquot at RT had a %RE of -29.4%. Two thirds of the aliquots meeting acceptance criteria will be considered sufficient to establish RT stability (see Exception 6). To determine the effect of continued freezing and thawing, samples that had been aliquoted and stored at -80°C were thawed for at least 30 minutes at RT and then overnight at -80°C. This was initially performed with validation samples for 5 cycles which were then thawed and analyzed (no additional aliquots to represent cycles 1 and 3 were stressed due to sample volume constraints). The validation samples did not meet acceptance criteria at the total of 6 cycles with a maximum %RE of -20.8%. As in 4°C and RT stability, Run07 used a single lot of rat CSF at one concentration. Three aliquots of the single rat CSF lot were subjected a total of 2 freeze/thaw cycles. Acceptance criteria was met with a maximum %RE of 6.7% See

Table 7 for all stressed and reference responses for 4°C, RT, and freeze thaw stability.

13.0 DATA ARCHIVING

Study documentation is archived per SOP QAU.Policy & Procedure.00017, Archiving of Laboratory and Study Materials. Documentation is initially archived on-site at Cambridge Biomedical facility, and subsequently transferred to long-term archival to an off-site archiving facility managed by a qualified vendor.

Electronic records are archived according to SOP QAU.Policy & Procedure.00061, Storage, Organization, and Archiving of Electronic Study Records. Electronic records are archived internally by Cambridge Biomedical on a local server with cloud-based remote data back-up, and remotely for long-term archiving in a cloud-based system with replication.

14.0 CONCLUSIONS

Criteria for intra-assay precision and establishment of limits of quantification were met as written in the method validation protocol. Inter-assay precision and parallelism data both suggest that a more reasonable expectation of variability in the assay across dilutions is a %CV of 30%. The acceptable %CV for QCs and samples will be updated to 30% before starting sample analysis. Also, the parallelism results are considered acceptable for the MRD of 8-fold, although the 8-fold diluted samples tend to give lower results compared to 16-fold and above diluted samples. The limits of quantitation for the assay will be Standard01 to Standard07 (40 ng/mL to 1.18 ng/mL). As expected of a protein that is so susceptible to problems from perturbation, the limits of short-term stability had to be slightly less to maintain assay integrity. Following the changes listed above to the method SOP, the assay described in CRO.SOP.00294 is considered suitable for the purpose of measuring prion protein in rat CSF.

15.0 DOCUMENT REVISION HISTORY

Amendment Number (Document Number/Version)	Document Section Amended	Change Made	Rationale for the Change
Original (CRO.Validation.01091 Version 1.0)	Not applicable	Not Applicable	New Document.

16.0 RESULT TABLES

Table 1:Run Summary

Run Summary					
Run#	Plate#	Date	Purpose	Analyst	Instrument
1	1	24-Jul-19	Intra-Assay Precision, Inter-Assay Precision	MRM	CB-1301
1b	1	06-Sep-19	Inter-Assay Precision	MRM	CB-1301
2	1	10-Sep-19	Inter-Assay Precision	MRM	CB-1303
3	1	12-Sep-19	Intra-Assay Precision, Inter-Assay Precision	MRM	CB-1301
4	1	20-Sep-19	Inter-Assay Precision	JM	CB-1303
5	1	21-Sep-19	Inter-Assay Precision, Parallelism	JM	CB-1303
6	1	26-Sep-19	Inter-Assay Precision, Short Term Stability, Parallelism Repeated	MRM	CB-1303
7	1	22-Oct-19	Inter-Assay Precision, Short Term Stability Repeated, Parallelism Additional Individuals	JMM	CB-1303

Table 2: Intra-Assay Precision

Intra-Assay Precision (Run03)			
Run	VS-H (ng/mL)	VS-M (ng/mL)	VS-L (ng/mL)
3	23.5	9.76	6.05
	24.3	10.5	6.25
	25.4	11.0	5.89
	24.4	10.3	6.37
SD	0.782	0.532	0.210
Mean	24.4	10.4	6.14
CV	3.20	5.12	3.42

Table 3: Inter-Assay Precision

Inter-Assay Precision (Run1b-06)			
Run	VS-H (ng/mL)	VS-M (ng/mL)	VS-L (ng/mL)
1b	19.8	7.37	4.12
2	23.4	8.53	4.81
3*	24.4	10.4	6.14
4	18.5	6.70	3.49
5	19.6	7.50	3.79
6	18.4	7.35	4.31
SD	2.57	1.32	0.95
Mean	20.7	8.0	4.4
CV	12.4	16.6	21.3

*Results reported for inter-assay precision of Run03 are the average of the 4 intra-assay precision determinations.

Table 4: Newly Established QC Ranges

Newly Established QC Ranges (Accuracy)			
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

Table 5: Limits of Quantitation

Calibration Curve (ng/mL)								
Nominal Conc	40.0	22.2	12.3	6.86	3.81	2.12	1.18	0
Run	Std01	Std02	Std03	Std04	Std05	Std06	Std07	Std08
1b	40.0	22.2	Masked	6.79	3.82	2.18	1.24	N/A
2	40.0	22.3	12.2	6.91	3.70	2.16	1.42	N/A
3	40.1	21.9	12.7	6.80	3.58	2.15	1.24	N/A
4	40.3	21.4	13.0	6.93	3.65	1.95	1.05	N/A
5	40.1	21.9	12.6	6.81	3.66	2.12	1.20	N/A
6	40.0	22.3	12.3	6.79	3.77	2.26	1.27	N/A
7	40.1	21.7	12.8	6.83	3.67	2.02	1.12	N/A
SD	0.107	0.319	0.320	0.0606	0.0796	0.103	0.117	N/A
Mean	40.1	22.0	12.6	6.84	3.69	2.12	1.22	N/A
CV	0.27	1.45	2.54	0.89	2.16	4.88	9.62	N/A
RE	0.181	-1.04	2.50	-0.339	-3.12	-0.040	3.35	N/A

LLOQ = 1.18 ng/mL, ULOQ = 40.0 ng/mL

Calibration Curve (Mean OD - Background)								
Run	Std01	Std02	Std03	Std04	Std05	Std06	Std07	Std08
1b	2.054	1.270	Masked	0.416	0.243	0.152	0.103	0.044
2	2.084	1.367	0.796	0.452	0.242	0.147	0.106	0.034
3	2.254	1.410	0.864	0.479	0.262	0.166	0.108	0.039
4	2.745	1.756	1.183	0.693	0.395	0.231	0.140	0.048
5	2.697	1.744	1.086	0.604	0.329	0.195	0.118	0.035
6	1.883	1.178	0.685	0.390	0.226	0.148	0.100	0.042
7	2.248	1.421	0.909	0.513	0.287	0.165	0.099	0.029
SD	0.326	0.222	0.185	0.108	0.060	0.031	0.014	0.007
Mean	2.28	1.45	0.921	0.507	0.283	0.172	0.111	0.039
CV	14.3	15.3	20.1	21.3	21.2	17.9	13.1	16.9

Table 6: Parallelism

Parallelism (Run05)			
Total Dilution Fold*	OD	Adjusted Result (ng/mL)	%RE
64	0.187	16.2	N/A
128	0.140	23.5	45.4
256	0.070		
512	0.047		
1028	0.054		
2056	0.038		
Run05 LLOQ	0.118		
Run05 ULOQ	2.697		
CV of results within LOQ		26.2	

*Least dilute parallelism sample on the plate had a total dilution of 64-fold instead of 8-fold due to dilution scheme on Run05 platemap in addition to 8-fold MRD. Parallelism repeated in Run06. Additional parallelism performed in Run07

Parallelism (Run06)			
Total Dilution Fold	OD	Adjusted Result (ng/mL)	%RE
8	0.567	80.5	N/A
16	0.399	111	38.4
32	0.212	112	38.6
64	0.131	122	51.7
128	0.093		
256	0.057		
Run06 LLOQ	0.100		
Run06 ULOQ	1.883		
CV of results within LOQ		16.9	

Parallelism (Run07)			
Total Dilution Fold	OD	Adjusted Result (ng/mL)	%RE
8	0.148	14.2	N/A
16	0.113	21.0	47.9
32	0.089		
64	0.056		
128	0.080		
256	0.069		
Run07 LLOQ	0.099		
Run07 ULOQ	2.248		
CV of results within LOQ		27.3	

Table 7: Short-Term Stability

Freshly Thawed (Nominal Values) Run06		
Validation Sample ID	Result (ng/mL)	CV
VS-H	18.44	18.4
VS-M	7.351	7.4
VS-L	4.31	4.3

Freshly Thawed (Nominal Values) Run07		
Validation Sample ID	Result (ng/mL)	CV
Ref Stab01	1.5	5.4
Ref Stab02	1.7	8.7
Ref Stab03	1.7	0.5

Room Temp Stability Run06			
Validation Sample ID	Result (ng/mL)	CV	RE
RT Stab H	7.5	2.8	-59.3
RT Stab M	4.8	3.8	-34.7
RT Stab L	2.4	2.9	-44.3

Room Temp Stability Run07			
Validation Sample ID	Result (ng/mL)	CV	RE
RT Stab01	1.4	3	-6.7
RT Stab02	1.6	0.5	-5.9
RT Stab03	1.2	2.9	-29.4

4°C Stability Run06			
Validation Sample ID	Result (ng/mL)	CV	RE
4C Stab H	10.4	0.7	-43.6
4C Stab M	5.8	1.5	-21.1
4C Stab L	3.5	6.4	-18.8

4°C Stability Run07			
Validation Sample ID	Result (ng/mL)	CV	RE
4C Stab01	1.6	2.2	6.7
4C Stab02	1.6	1.4	-5.9
4C Stab03	1.7	1	0.0

Freeze/Thaw Stability (5 cycles) Run06			
Validation Sample ID	Result (ng/mL)	CV	RE
FT 5 Stab H	14.6	2.1	-20.8
FT 5 Stab M	6.6	3.1	-10.2
FT 5 Stab L	3.9	5.2	-9.5

Freeze/Thaw Stability (2 cycles) Run07			
Validation Sample ID	Result (ng/mL)	CV	RE
FT 2 Stab01	1.6	3.5	6.7
FT 2 Stab02	1.7	0.5	0.0
FT 2 Stab03	1.7	4.6	0.0

17.0 LIST OF ATTACHMENTS

17.1 Attachment A – Assay Performance Summary

17.1 Attachment A – Assay Performance Summary

Parameter	Expectation	Observed Performance
Intra-Assay Precision	CV ≤ 20% at VS-H, VS-M, VS-L	VS-H: 3.20% VS-M: 5.12% VS-L: 3.42%
Inter-Assay Precision	CV ≤ 30% at VS-H, VS-M, VS-L	VS-H: 12.4% VS-M: 16.6% VS-L: 21.3%
Accuracy	± 3 SD from average back calculated result of VS-H, VS-M, VS-L	VS-H: 13.0 - 28.4 ng/mL VS-M: 4.00 – 11.9 ng/mL VS-L: 1.61 – 7.28 ng/mL
Limits of Quantitation	Lowest and highest standards with CV and RE ≤ 25%	Std01: CV = 0.27%, RE = 0.181% Std07: CV = 9.62%, RE = 3.35%
Parallelism	CV ≤ 30% for all concentrations within the LOQ	Run05 (64 to 128-fold): 26.2% Run06 (8 to 64-fold): 16.9% Run07 (8 to 16-fold): 27.3%
4°C Stability	RE ≤ 20% for at least 2/3 of aliquots per lot of CSF tested	100% of aliquots pass, CV of -5.9 to 6.7%
RT Stability	RE ≤ 20% for at least 2/3 of aliquots per lot of CSF tested	67% of aliquots pass, CV of -6.7 to -5.9%. Failing aliquot CV = -29.4%
Freeze Thaw Stability	RE ≤ 20% for at least 2/3 of aliquots per lot of CSF tested	100% of aliquots pass, CV of 0 to 6.7%