



Title: Method Validation of the SARS-CoV-2 mNeonGreen Virus Microneutralization Assay

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Title: Method Validation of the SARS-CoV-2 mNeonGreen Virus Microneutralization Assay

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Title: Method Validation of the SARS-CoV-2 mNeonGreen Virus Microneutralization Assay

SYNOPSIS

This report describes the validation of the SARS-CoV-2 mNeonGreen Virus Microneutralization Assay (SARS-CoV-2 mNG NT) used for the detection of serum antibodies capable of neutralizing SARS-CoV-2. The assay readout of 50% virus neutralization titer was validated per protocol VR-MVP-10074.

- Based on dilutional linearity and assay precision, the lower limit of quantitation (LLOQ) was determined to be a titer equal to 41 and the upper limit of quantitation (ULOQ) was determined to be a titer equal to 3,187. Samples with titers greater than the ULOQ may be pre-diluted before testing to yield titers within the validated assay range.
- Intermediate precision assessment demonstrated overall assay variability of 26.5% RSD.
- Serum samples are run in replicate in the SARS-CoV-2 mNG NT. Based on the assay performance, it was determined that the replicate titer ratio for a sample must be less than or equal to 2.55 (sample extravariability rule).

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Title: Validation Report for the SARS-CoV-2 mNeonGreen Virus Microneutralization Assay

Study Number: N/A

Functional Area: Vaccine Research and Development

Test Facility: Hackensack Meridian Health
Nutley, NJ 07110

Study/Testing Initiation Date: 01-Dec-2020

Study/Testing Completion Date: 20-Jan-2021

1. OBJECTIVES

This report describes the validation results for the SARS-CoV-2 mNeonGreen Virus Microneutralization Assay (SARS-CoV-2 mNG NT) used for the detection of serum antibodies capable of neutralizing SARS-CoV-2. The assay readout of 50% virus neutralization titer was validated per protocol VR-MVP-10074.¹ This validation provides documented evidence that the SARS-CoV-2 mNG NT is suitable for its intended use when performed in accordance with standard operating procedures by qualified personnel.

2. INTRODUCTION

The SARS-CoV-2 mNG NT is a biofunctional assay that measures neutralizing antibodies against SARS-CoV-2. The SARS-CoV-2 mNG virus is derived from the USA_WA1/2020 strain that had been rescued by reverse genetics and engineered to contain a mNeonGreen (mNG) reporter gene in open reading frame 7 of the viral genome that produces green fluorescence upon productive infection of cells.² This reporter virus generates similar plaque morphologies and indistinguishable growth curves from wild-type virus.³

This assay is described in the test method VR-TM-10298.⁴ Briefly, serially diluted test serum samples are mixed with SARS-CoV-2 mNG virus in a 96-well plate to allow virus-specific antibodies to bind to the virus. This serum-virus mixture is then transferred onto a Vero cell monolayer and incubated overnight to allow for infection by non-neutralized virus. Productive viral infection is detected by enumerating green-fluorescent viral foci using a cell-imaging reader. The total number of cells per well is calculated by enumerating Vero cell nuclei stained blue with Hoechst 33342. An infection ratio is then calculated for each well, whereby the total number of virus infected (green) cells is divided by the total number of cells present (blue nuclei). A sample titer is defined as the reciprocal serum dilution at which a specific percentage of the virus is neutralized, eg, 50%, 80% or 90% (termed “Titer Determining Value”, TDV).

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3. GLOSSARY

Table 1. Terms and Definitions

Term	Definition
Assay range	Range of neutralization titers that can be measured in the assay with acceptable dilutional linearity and precision.
COVID-19	Coronavirus Disease 2019
DL	Dilutional linearity
GMT	Geometric Mean Titer
LLOQ	Lower Limit of Quantitation
LOD	Limit of Detection
MDP	Master Dilution Plate
NHS_dep	Normal Human Serum-Antibody Depleted
NT	Microneutralization Assay
QCS	Quality Control Sample
Replicate	An independent determination of an assay result; the geometric mean of two replicate titers is the reportable result for a sample tested in the SARS-CoV-2 mNG NT.
RSD	Relative Standard Deviation
SARS-CoV-2	Severe acute respiratory syndrome coronavirus 2; the etiologic agent of COVID-19
SARS-CoV-2 mNG NT	96-well manual microneutralization assay for the detection of functional antibodies to SARS-CoV-2 using the mNeonGreen reporter virus.
SAS	Programming language and integrated software solution-set proprietary to SAS® (the company) that enables the coding of the various tasks associated with handling datasets.
SOP	Standard Operating Procedure
TDV	Titer Determining Value; the threshold value in percent of measured viral green particle counts that is used to report sample titers. Titers may be reported at 50%, (b) (4) 90% TDV.
Titer	Inverse of serum dilution required to neutralize a specific percentage of the input virus (eg 50% TDV).
TM	Test Method
ULOQ	Upper Limit of Quantitation
	(b) (4)
Vero	African green monkey kidney epithelial cell line

4. MATERIALS AND METHODS

General material supplies, reagents and equipment used are listed in SOPs VR-TM-10298,⁴ VR-SOP-LC-11299,⁵ VR-SOP-LC-11294,⁶ and VR-SOP-LC-11287.⁷

4.1. Critical Reagents

Critical reagents used for the validation assays are listed in [Table 2](#).

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Table 2. Critical Reagents for the SARS-CoV-2 mNG NT Validation

Reagent	Name/Lot#
Virus Stock	SARS-CoV-2-mNG Lot 5 P2 Vero E6 (b) (4) (b) (4)
Cell Line	Vero cells; ATCC CCL81, used from passage 134 to 155

4.2. Quality Control Samples

Quality control samples (QCS) were generated from pools of (b) (4) (b) (4) QCS lower and upper titer limits were established prior to the start of validation as documented in VR-RGR-RQ-10781⁸ and are listed in Table 3.

Table 3. Quality Control Sample Specification Limits

QCS	Lower Specification Limit	Upper Specification Limit
(b) (4)		

4.3. Negative Serum Diluent

(b) (4) was used as a negative serum diluent for dilutional linearity testing during this validation.

4.4. Validation Serum Sample Panels

Panels comprised of serum samples from either (b) (4) (b) (4) were selected to be used in the evaluation of assay dilutional linearity and precision. Convalescent serum was obtained from (b) (4) (b) (4) Negative samples were collected from donors in 2013 (pre-COVID-19). All sera were heat-inactivated for 30 min at 56°C prior to testing.

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Table 4. Validation Serum Sample Panels

Intended Purpose	Sample ID	Serum Source
Dilutional Linearity Panel	A large grey rectangular redaction box covers the Sample ID and Serum Source columns for all three rows. The text "(b) (4)" is printed in large black font in the center of the redaction.	
Precision Panel		
LOD Assessment		

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5. EXPERIMENTAL DESIGN

Validation of the SARS-CoV-2 mNG NT was performed as described in the validation protocol, VR-MVP-10074.¹ The assay readout of 50% virus neutralization titer (50% TDV) was validated; (b)(4) 90% neutralization datasets were collected for exploratory purposes only (VR-VTR-10742⁹). Dilutional linearity and precision were evaluated in separate assay runs according to the schedule shown in Table 5. The factorial design includes (b)(4) assay runs, completed on (b)(4) different days, utilizing (b)(4) different sets of sample master dilution plate (MDP) preparations, performed by (b)(4) different analysts in order to quantify the contribution of these factors to the intermediate precision. Sample MDPs are prepared in deep-well 96-well plates and each are of sufficient volume to stamp out sample assay plates on each of the (b)(4) assay days in the experimental design (refer to Supportive Figure 12.1 for the assay design schematic).

- For the dilutional linearity assessment, (b)(4) serum samples were tested (b)(4) independently diluted (b)(4)
- For the precision assessment, (b)(4) (b)(4) serum samples were tested (b)(4) (n=(b)(4)) and analyzed along with the (b)(4) dilutional linearity samples (n=8).
- For the evaluation of the assay near the LOD, (b)(4) presumed negative serum samples were tested (b)(4).

The assay schedule for validation testing is shown in Table 5.

Table 5. Example Validation Run Schedule

Assay	MDP Set ^a	Day	Analyst
(b)(4)	(b)(4)	(b)(4)	(b)(4)

6. STATISTICAL ANALYSIS

All analyses of titer data described in the statistical analysis sections are based on the reportable geometric mean titer (GMT). The reportable titer is the (b)(4) (b)(4). Unless indicated otherwise, the word titer will be understood to be reportable GMT at 50% TDV. All ranges and/or limits determined based on the statistical analyses will be applied for all non-clinical and clinical testing moving forward.

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(b)(4)

All statistical analyses, including (b)(4), were performed using SAS® version 9.4.

6.1. Dilutional Linearity

Each dilutional linearity sample was tested at (b)(4) dilutions (b)(4) and at dilutions of (b)(4) in (b)(4) assay runs. For each assay run, a dilution-adjusted titer was calculated for each of the (b)(4) dilutions. (b)(4)

(b)(4)

10

(b)(4)

6.2. Precision

Sample precision refers to the closeness of 2 or more measurements of a sample to each other and was reported as %RSD. The (b)(4) precision samples were tested (b)(4) in (b)(4) independent runs (refer to [Table 5](#)). The evaluation of precision consisted of using the titers of the (b)(4) samples from the precision panel combined with the (b)(4) samples prepared for dilutional linearity.

(b)(4)

(b) (4)

6.3. Limits of Quantitation

The lower and upper limits of quantitation (LLOQ and ULOQ, respectively) were defined by the range of titers that have acceptable dilutional linearity and precision. The most conservative values from the lower and upper titer limits from dilutional linearity and precision, as described in [Section 6.1](#) and [Section 6.2](#), respectively, were used to determine the assay range. The assay range is bounded by the LLOQ and ULOQ.

6.4. Assay Intermediate Precision

To evaluate the intermediate precision of the assay, (b) (4) was performed using the model below that (b) (4). Only titers within the assay range (limits of quantitation) were used for this analysis.

The estimated overall assay precision was based on (b) (4) using the following model which (b) (4)

(b) (4)

(b) (4)

6.5. Limit of Detection

The limit of detection (LOD) was set to the lowest possible dilution tested in the assay (titer of 20). The LOD was used during assay development in lieu of an established LLOQ. Since the LLOQ was determined through the validation experiments described here, the LLOQ will be used in place of the LOD. However, to demonstrate assay performance near the LOD, 8 presumed negative samples were tested (b)(4) in each of the (b)(4) assay runs, for a total of up to (b)(4) reportable titers. The data is presented as a descriptive measure of assay performance.

6.6. Extravariability of Replicates

As defined in the data review SOP, VR-SOP-LC-11293,¹¹ reportable titers are the geometric means of the (b) (4)

(b) (4)

7. RESULTS AND DISCUSSION

Assay validation runs were performed by qualified analysts from 01-Dec-2020 through 20-Jan-2021, inclusive. The 9 assay runs were performed to assess dilutional linearity, precision, assay performance near the LOD, and to determine LLOQ and ULOQ. The raw titer data for the dilutional linearity and precision experiments are listed in attachments VR-MVR-10083-ATT01¹² and VR-MVR-10083-ATT02,¹³ respectively. LOD titer data are listed in VR-MVR-10083-ATT03.¹⁴ Documentation of analyst training on the validation protocol is provided in VR-MVR-10083-ATT04.¹⁵

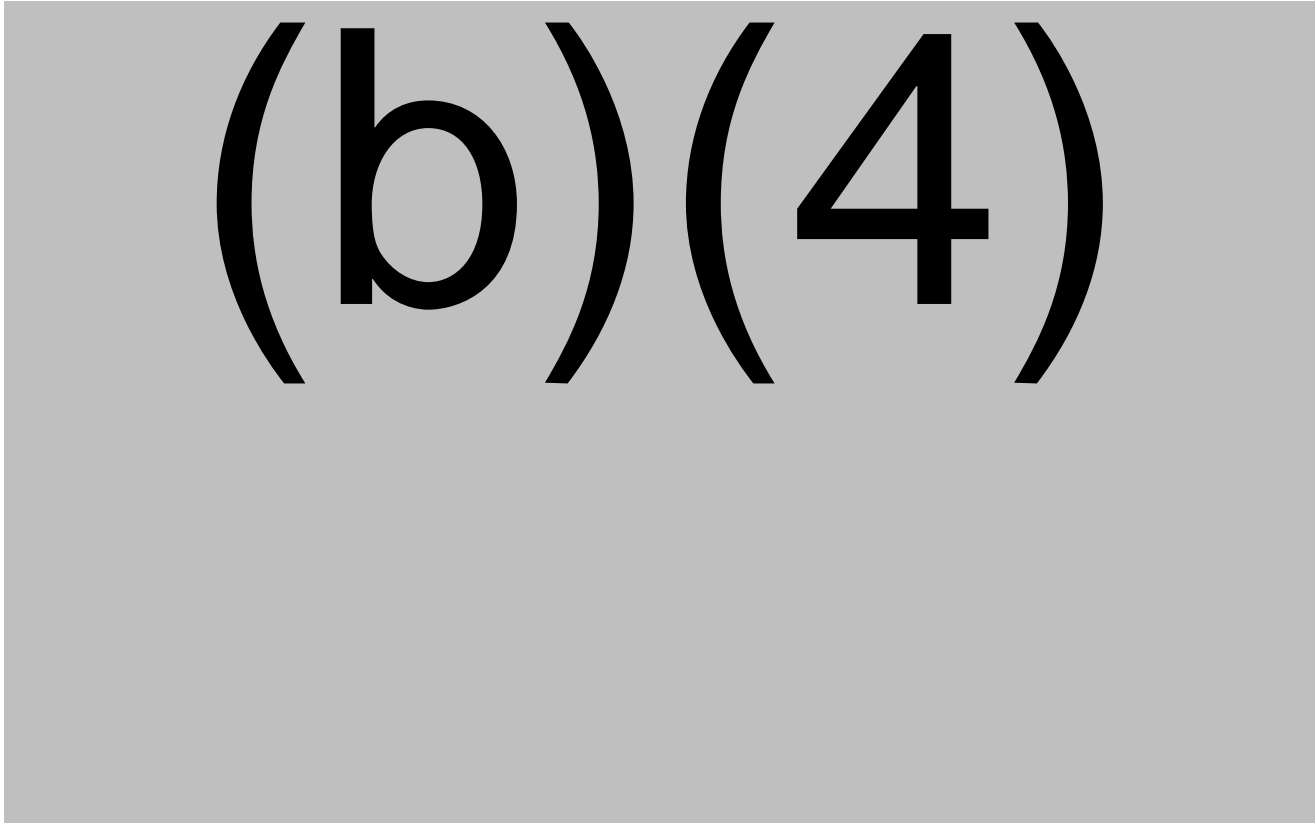
7.1. Dilutional Linearity

A sample shows dilutional linearity when its titer changes in proportion to its dilution. Each sample replicate was evaluated (b)(4) and at dilutions of (b)(4) (b)(4)

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For each of the (b)(4) dilutional linearity samples, (b)(4) (b)(4) (b)(4) (Figure 1). Supportive Figure 12.2 shows this data for all (b)(4) individual samples from the (b)(4) runs plotted together.

Figure 1. Dilutional Linearity Results Plot



The dilutional linearity data displayed is based on (b)(4) samples, each assayed (b)(4) dilutions, tested in (b)(4) independent runs. The dilutional linearity (b)(4)

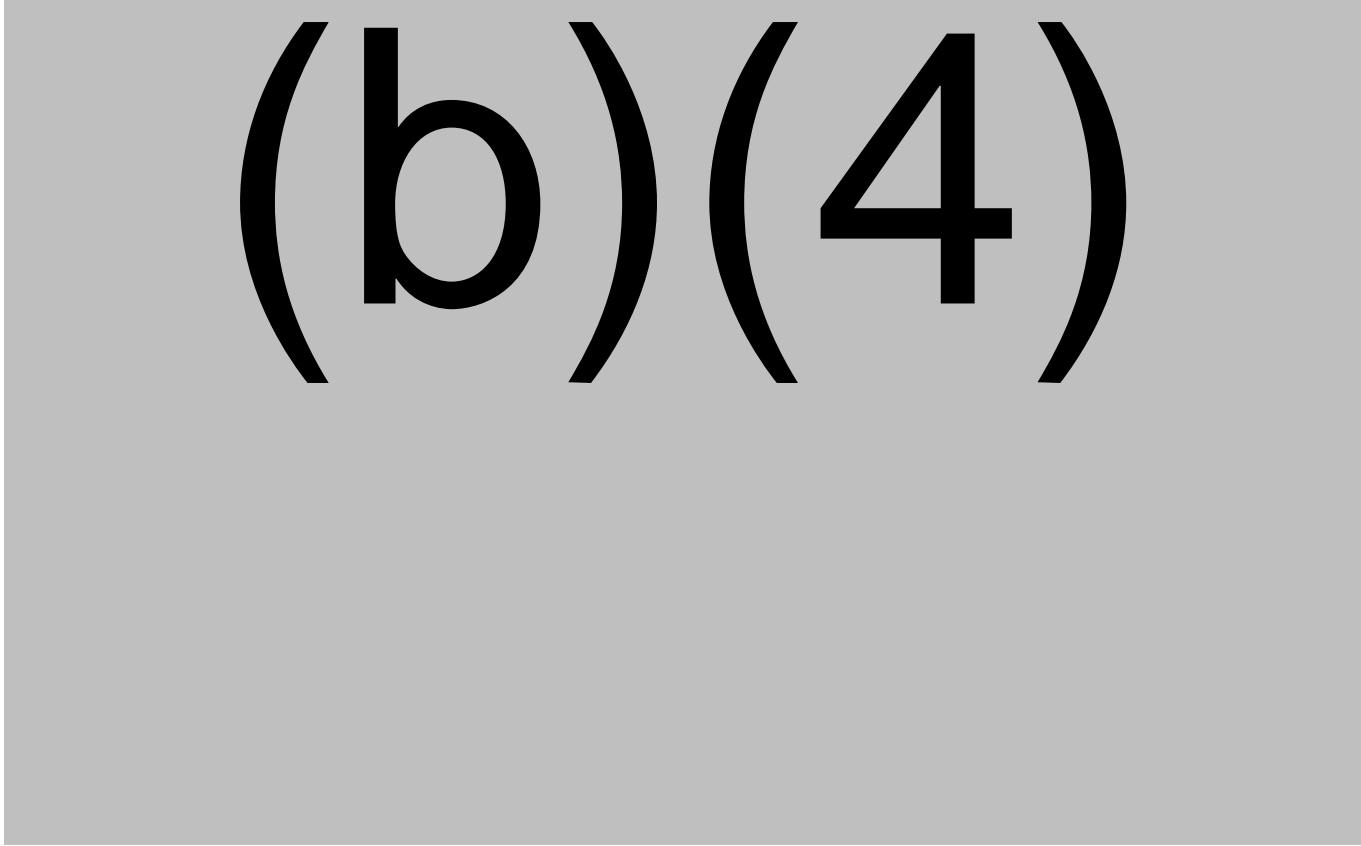
7.2. Precision

The assay precision was calculated (b)(4) (b)(4) (b)(4) (b)(4). Precision results for individual samples are displayed in Supportive Table 11.1.

(b)(4) (b)(4) Acceptable precision (b)(4) was demonstrated between titers of 41 and 3,187 (Figure 2).

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Figure 2. Precision Results Plot



The precision data displayed is based on (b)(4) precision samples, tested twice in (b)(4) independent runs, and (b)(4) (b)(4), tested once in (b)(4) independent runs. The precision lower and upper titer limits are 41 and 3,187, respectively.

7.3. Limits of Quantitation

(b)(4)
Table 6 displays the final assay range bounded by the LLOQ and ULOQ.

Table 6. Final Assay Range

Dilutional Linearity Range	Precision Range	Final Assay Range	
		Lower	Upper
(b)(4)	(b)(4)	41	3,187

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7.4. Assay Intermediate Precision

The intermediate precision of the assay was evaluated using the (b)(4) (b)(4) as described in Section 6.4 and the results are summarized in Table 7. (b)(4)

The total %RSD is the estimated intermediate precision and the (b)(4) (b)(4)

Table 7. (b)(4) Samples (b)(4)

(b)(4) (4)	%RSD
	(b)(4)
	26.5

7.5. Limit of Detection

The limit of detection (LOD) was set to the lowest possible dilution tested in the assay (titer of 20). To demonstrate assay performance near the LOD, (b)(4) negative samples were tested (b)(4) in each of the (b)(4) assay runs, for a total of up to (b)(4) titers or a maximum number of (b)(4) titers per sample. The number of reportable titers for each sample as well as the percent that the sample returned a negative titer is listed in Table 8.

Table 8. Assay Performance Near the LOD

Sample	N of Results ^a	% Negative ^b
(b)(4) (4)		100
		100
		100
		100
		100
		100
		100
		100

- a. There were (b)(4) titers measured out of the (b)(4) titers possible. Sixteen titers were not resolved due to failed assay suitability requirements on the plate.
- b. A sample is considered negative if its titer is less than the LOD of 20 (ie, the lowest measurable titer).

7.6. Extravariability of Replicates

An upper limit for acceptable replicate titer ratios was computed from titer pairs by the method defined in Section 6.6. This ratio limit was 2.55, indicating good agreement between titers of replicate pairs.

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8. CONCLUSION

The results documented in this validation report for the SARS-CoV-2 mNG NT provide evidence that the assay is validated and suitable for its intended use in testing clinical, epidemiological, and non-clinical study samples. The assay demonstrated dilutional linearity and precision that met predefined acceptance criteria. The LLOQ for the assay is a titer of 41 and the ULOQ a titer of 3,187. Samples with titers greater than the ULOQ may be pre-diluted in assay buffer before testing to yield titers within the validated assay range. The performance of the assay near the LOD is acceptable.

9. DEVIATIONS

- During statistical analysis of the validation dataset, it was observed that SAS was inappropriately assigning a final titer to samples with only one valid replicate titer. This occurred 4 times in the validation and in each case the assay result was overridden to the (b)(4). These assay results were then appropriately excluded from the final data set and validation analysis. This deviation was recorded in Laboratory Deviation Report VR-LDR-12674. The SAS data system is being updated for future clinical testing.
- Section 5.4 of the validation protocol (VR-MVP-10074¹) incorrectly stated that the (b)(4) diluent would be obtained from (b)(4), however it was prepared at Pfizer.
- Section 7.7 of the validation protocol (VR-MVP-10074¹) stated that a replicate titer ratio limit of (b)(4) be applied to all replicate titer pairs during validation. However, a more conservative limit of (b)(4) was applied after preliminary examination of the validation data.

10. REFERENCES

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7. VR-SOP-LC-11287: Preparation of Samples for SARS-CoV-2 Neutralization Assay Using the (b)(4) Workstation.
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12. [VR-MVR-10083-ATT01: Dilutional Linearity Data Listing for VR-MVR-10083.](#)
13. [VR-MVR-10083-ATT02: Precision Data Listing for VR-MVR-10083.](#)
14. [VR-MVR-10083-ATT03: LOD Data Listing for VR-MVR-10083.](#)
15. [VR-MVR-10083-ATT04: Training Documentation VR-MVP-10074 for VR-MVR-10083.](#)

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11. SUPPORTIVE TABLES

11.1. Precision by Individual Sample

Data Source	Sample	Dilution	N	GMT	(b)(4)	%RSD
Precision						
Dilutional Linearity						

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12. SUPPORTIVE FIGURES

12.1. Assay Design Schematic

(b) (4)

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12.2. Dilutional Linearity Plot by Individual Sample

(b) (4)

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