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

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

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

SYNOPSIS

This protocol describes the processes and requirements for 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. This assay may be used to evaluate clinical trial sera and support epidemiological and non-clinical studies. The following parameters will be investigated:

1. Dilutional Linearity
2. Precision
3. Intermediate Precision (inter-assay variability)
4. Lower and Upper Limits of Quantitation (LLOQ and ULOQ)
5. Limit of Detection (LOD)
6. Extravariability Ratio for Replicate Titers

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

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1. OBJECTIVES

This protocol describes the processes and requirements for 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. This validation will provide 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.[1] This reporter virus generates similar plaque morphologies and indistinguishable growth curves from wild-type virus.[2]

This assay is described in the test method VR-TM-10298.[3] 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 visualizing Hoechst 33342 stained Vero cell nuclei, which are blue. 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).

3. GLOSSARY

Table 1. Terms and Definitions

Term	Definition
Assay range	Range of antibody titers that can be measured in the assay with acceptable dilutional linearity and precision.
BSL-3	Biosafety Level 3
COVID-19	Coronavirus Disease 2019
DL	Dilutional linearity
GDMS	Global Document Management System
GMT	Geometric Mean Titer
GxP	Good Practice Quality Guidelines
IR	Infection ratio; the number of virus infected cells (green) divided by the total number of cells present (blue)
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
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) or 90% TDV.
TM	Test Method
ULOQ	Upper Limit of Quantitation
(b) (4)	
Vero	African green monkey kidney epithelial cell line

4. ROLES AND RESPONSIBILITIES

Table 2. Roles and Responsibilities

Role	Responsibilities
Assay Analyst	• Read, understand, and complete training on the applicable Standard Operating Procedures (SOP) and Test Methods (TM) used to perform the SARS-CoV-2 mNG NT. • Execute this validation protocol.
(b) (4) **Analyst**	• Read, understand, and complete training on the applicable Standard Operating Procedures (SOP) and Test Methods (TM) used to prepare the serum dilution assay plates used in the SARS-CoV-2 mNG NT. • Prepare serum dilution assay plates for use in executing this protocol.

Table 2. Roles and Responsibilities

Role	Responsibilities
Reviewer	• Read, understand, and complete training on the applicable Standard Operating Procedures (SOP) and Test Methods (TM) used to perform the SARS-CoV-2 mNG NT. • Read and understand this validation protocol. • Review the SARS-CoV-2 mNG NT documentation to ensure completeness of data entry and compliance with relevant policies. Direct the Assay Analyst to correct documentation errors. • Consult with a Supervisor or Senior Manager if there are any questions related to the procedure itself, the serum samples, atypical events or protocol deviations that may occur.
Supervisor	• Ensure that the Assay Analyst and (b) (4) Analyst executing this protocol are trained on the appropriate SOPs and TMs. • Review and evaluate data generated during execution of this validation protocol. • Author validation protocol and/or reports. • Ensure that validation protocol and report are entered into GDMS.
Senior Manager	• Review and approve this validation protocol. • Ensure the accurate execution of this validation protocol. • Review and evaluate data generated during execution of this validation protocol.
Statistician	• Works with the Supervisor to create a final experimental design for this protocol prior to the initial review workflow. • Analyzes the final data supplied by the Supervisor. • Collaborates to coauthor the validation report.

All personnel and positions referred to in this procedure are considered to have an alternate. An alternate must ensure that they are trained and knowledgeable with the process.

5. MATERIALS AND METHODS

- General material supplies, reagents and equipment used in this protocol are listed in SOPs VR-TM-10298[3] (Assay Test Method), VR-SOP-LC-11299[4] (Vero cell culture), and VR-SOP-LC-11287[5] (Sample Preparation on (b) (4) Workstation).

- A table listing of critical reagent lot numbers will be provided in the Validation Report.

5.1. Viral Stock

The SARS-CoV-2 mNG viral stock was produced as described in VR-SOP-LC-11294,[6] aliquoted into single-use vials, and frozen at -80°C.

5.2. Cell Culture

The Vero cell line (CCL81) was purchased from ATCC and used to generate an in-house cell bank for long-term use. Cells are passaged according to VR-SOP-LC-11299[4] for up to (b) (4) cell passages.

5.3. Quality Control Samples

Quality control samples (QCS) were generated to monitor routine assay performance and will be used to determine the fitness of assay runs during validation. A low and a medium titered QCS will be run on alternating assay plates, (b) (4) QCS per plate, on each plate of a given run. The QCS were generated from (b) (4), (b) (4), and lower and upper specification limits will be established and documented prior to the start of validation.

5.4. Negative Serum Diluent

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

5.5. Validation Sample Panels

Panels comprised of either (b) (4) were selected to be used in the evaluation of assay dilutional linearity and precision. (b) (4) (b) (4) serum was obtained from (b) (4) (b) (4) Negative samples were collected (b) (4) All sera were heat-inactivated for 30 min at 56°C prior to testing. A table listing of sample source information will be provided in the Validation Report.

5.6. Assay Methods

The Test Method, VR-TM-10298,[3] describes the procedure to perform the SARS-CoV-2 mNG NT for detection of SARS-CoV-2 neutralizing antibodies. This method was qualified as described in VR-MQR-10214.[7]

The SARS-CoV-2 mNG NT is a three day manual 96-well assay. On Day 0, Vero CCL-81 cells are seeded into 96-well tissue-culture treated read-out plates (ROPs). On Day 1, serial dilutions of heat-inactivated test sera are incubated with the SARS-CoV-2 mNG virus to allow any virus-specific antibodies to bind to the virus. The serum-virus mixture is then transferred onto the Vero cell monolayer and incubated for (b) (4) hours to allow for infection by non-neutralized virus. On Day 2, productive viral infection is enumerated by visualizing fluorescent viral foci on a Cytation-7 Cell Imaging Multi-Mode Reader. Total number of cells per well is calculated by visualizing Hoechst 33342 stained Vero cell nuclei, which are blue.

Using a validated custom SAS application, 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 serum titer is defined as the reciprocal serum dilution at which a specific percentage (eg, 50%, (b) (4) or 90%) of virus is neutralized compared to the no serum control (100% infection, 0% neutralization). This value is referred to as the titer determining value (TDV).

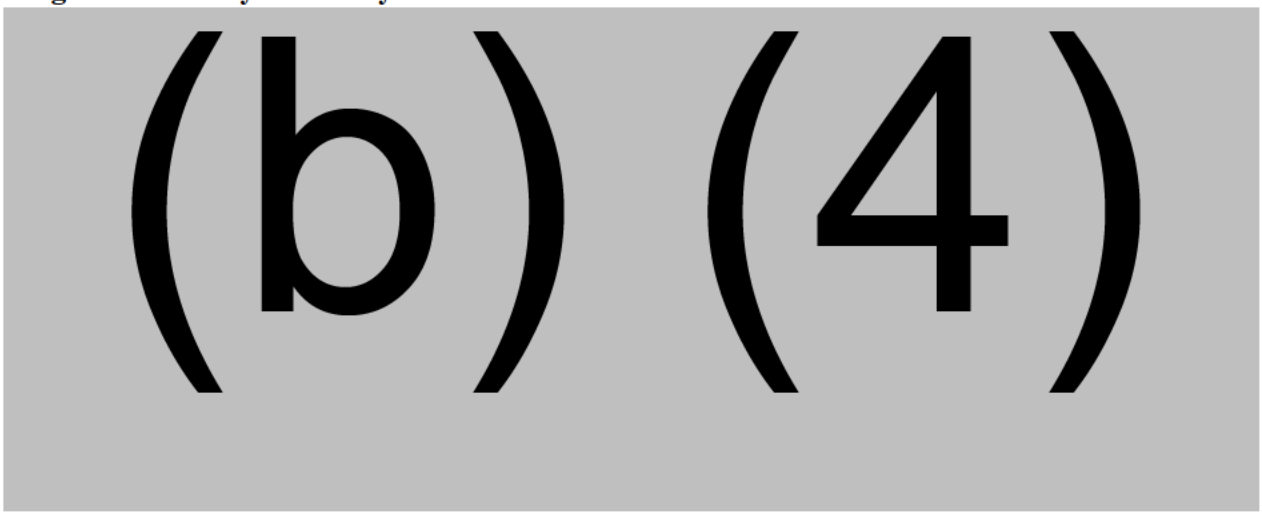
For routine clinical testing, titers corresponding to a TDV of 50% are reported, however (b) (4) and 90% are also collected for exploratory purposes. A titer is calculated for each of the (b) (4)

(b) (4) of a sample on a plate and the geometric mean titer (GMT) of the (b) (4) is reported as the final sample titer.

A clinical SARS-CoV-2 mNG NT assay consists of between (b) (4) assay plates per run (batch). Sample plates are prepared using a (b) (4) liquid handling workstation. The assay plate layout (Figure 1) accommodates (b) (4) A maximum of (b) (4) samples may be tested in a (b) (4) plate batch. A (b) (4) (b) (4) of a given run to assess plate and run suitability.

The initial serum dilution for the SARS-CoV-2_NT is 1:20, which, along with a (b) (4) (b) (4) series, enables a potential plate titer range of 20 to (b) (4). Samples that receive titers greater than (b) (4) are retested after pre-dilution in assay buffer. Samples that fail to neutralize at the TDV when tested at the lowest serum dilution of 1:20 are reported to have a neutralizing titer of (b) (4)

Figure 1. Assay Plate Layout



6. PROTOCOL DESIGN

6.1. Validation Parameters

This validation is intended to demonstrate consistent and acceptable performance of the SARS-CoV-2 mNG NT. The following parameters will be investigated:

1. Dilutional Linearity
2. Precision
3. Intermediate Precision (inter-assay variability)
4. Assay Range (LLOQ and ULOQ)
5. Limit of Detection (LOD)
6. Extravariability Ratio for Replicate Titers

6.2. Design Summary

Validation of the SARS-CoV-2 mNG NT will consist of a series of assay runs to address the parameters listed in Section 6.1. All assay runs will be performed according to test method VR-TM-10298.[3] Data generated from this validation protocol will be reviewed and accepted according to VR-SOP-LC-11293.[8] Of note, the assay readout of 50% virus neutralization (50% TDV) will be validated according to this protocol; (b) (4) and 90% TDVs will be collected for exploratory purposes only.

This validation will consist of at least (b) (4) runs for dilutional linearity and (b) (4) runs for precision. Serum samples will be prepared in Master Dilution Plates (MDPs). There will be (b) (4) sets of MDPs (b) (4) that will be used to prepare dilutional linearity assay plates, and (b) (4) sets that will be used to prepare precision assay plates. (b) (4) assay runs will be performed from each set of MDPs for a total of (b) (4) runs for precision and (b) (4) runs for dilutional linearity.

Additional assay runs may be added to the validation, as needed, to ensure a minimum number of observations for each sample are obtained to compensate for partial or complete assay failures based on system suitability criteria.

The titer data from the validation experiments are based on the reportable geometric mean titer (GMT). The reportable titer, also known as the final titer, is the geometric mean of the (b) (4) and, unless indicated otherwise, the word titer will be understood to mean GMT.

6.2.1. Design of Dilutional Linearity and Precision Experiments

Assessments of dilutional linearity and precision will be performed with data obtained from

(b) (4)

(b) (4) Refer to Validation Run Plate Map in Supportive Table 11.2.

- For the evaluation of negative sera, (b) (4) serum samples with presumed negative titers will be tested (b) (4). The negative samples will be split across the dilutional linearity and

precision runs as shown in the Validation Run Plate Maps in Supportive Table 11.1 and 11.2, respectively.

Table 3. Validation Run Schedule

(b) (4)

6.2.2. Dilutional Linearity Samples

(b) (4)

(b) (4) (refer to Supportive Table 11.1, for Dilutional Linearity Validation Run Plate Map).

6.2.3. Precision Samples

(b) (4)

(b) (4) (refer to Supportive Table 11.2, for Precision Validation Run Plate Map).

6.2.4. Negative Samples

A panel of (b) (4) negative sera will be tested (b) (4) in a total of (b) (4) assays generating a maximum of (b) (4) reportable titers per sample. The negative serum samples are distributed across the Dilutional Linearity and Precision runs according the Validation Run Plate Maps in Supportive Table 11.1 and 11.2, respectively. The samples will be used to descriptively evaluate the assay performance near the LOD.

7. STATISTICAL ANALYSIS

7.1. Log Transformation

(b) (4)

All analyses in subsequent statistical analysis sections are based on the reportable geometric mean titer (GMT).

7.2. Dilutional Linearity

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

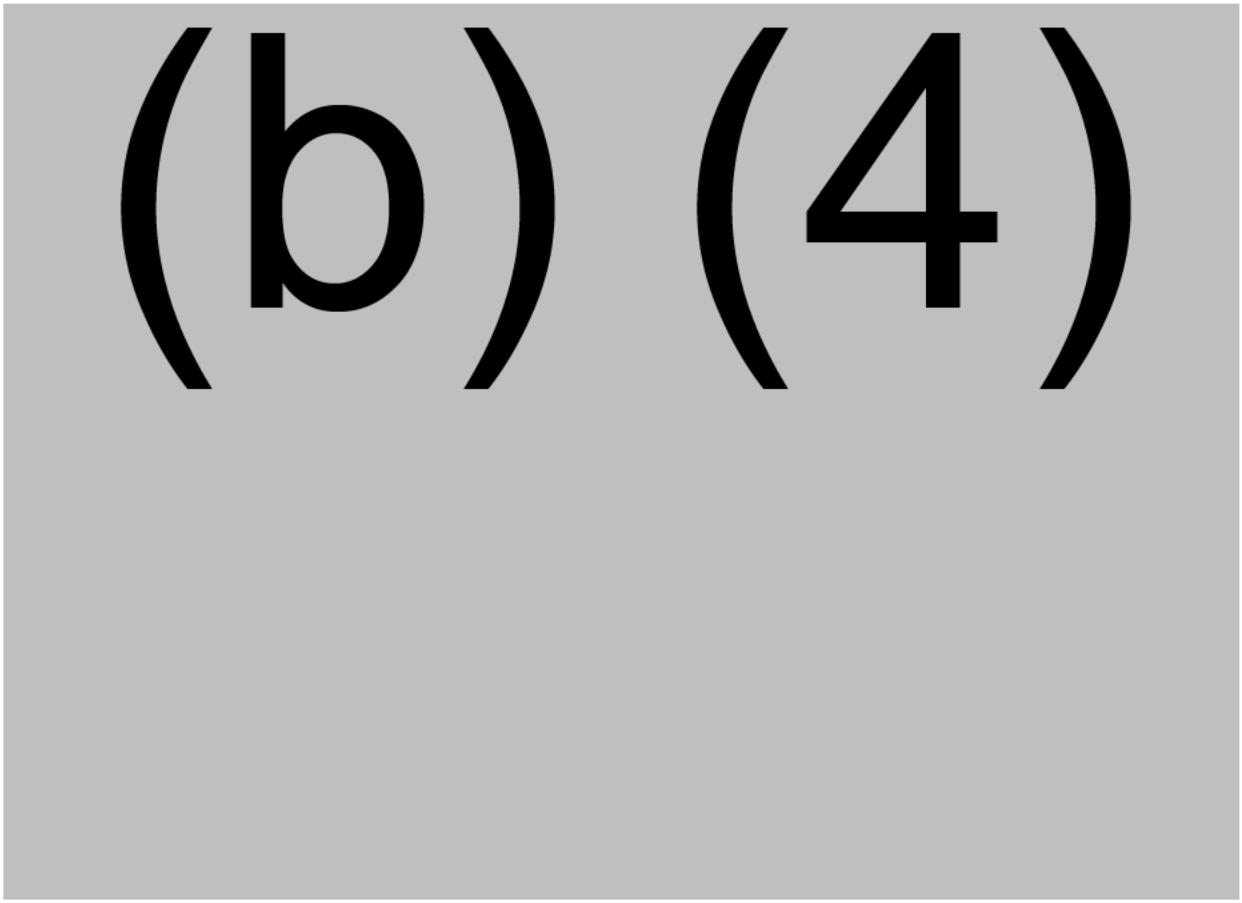
(b) (4)

(b) (4)

7.3. Precision

The evaluation of precision will consist of using the reportable titers of the (b) (4) samples from the precision panel combined with the (b) (4) samples prepared for dilutional linearity (Section 6.2.2).

(b) (4)



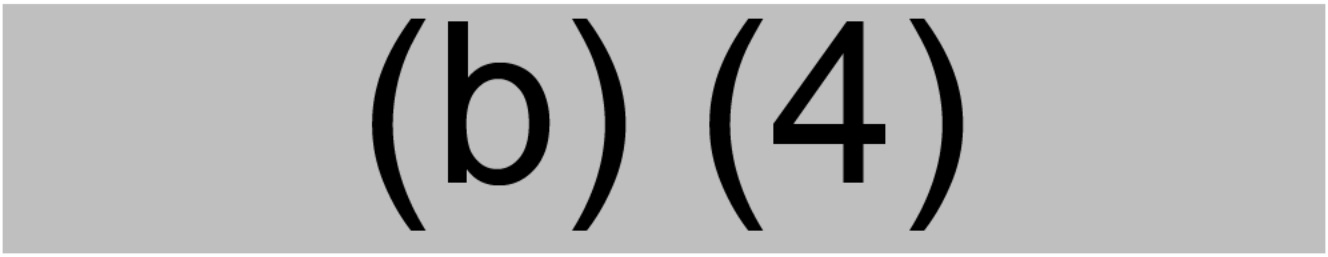
7.4. Limits of Quantitation

The lower and upper limits of quantitation (LLOQ and ULOQ, respectively) are 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 7.2 and Section 7.3, respectively, will be used to determine the assay range. The assay range is bounded by the LLOQ and ULOQ.

7.5. Assay Intermediate Precision

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

Estimates of the overall assay precision will be based on (b) (4) using the following model which (b) (4)



(b) (4)

7.6. Limit of Detection

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

7.7. Extravariability of Replicates

As defined in the data review SOP, VR-SOP-LC-11293,[8] reportable titers are the geometric means of the (b) (4)

(b) (4)

7.8. Titer Reporting Range

The validated assay range of the SARS-CoV-2 mNG NT will be the range of sample titers that are shown to have suitable assay performance. This assay performance will define the lower and upper limits of quantitation (LLOQ and ULOQ, respectively) for SARS-CoV-2 neutralizing antibodies, as described in Section 7.4. The maximum limits for the titer reporting range without pre-dilution of test sera are neutralization titers between 20 and (b) (4), since the plate design can determine titers in this range only. The LLOQ and ULOQ determined upon execution of this validation protocol may further limit the titer reporting range for clinical serum samples tested in the SARS-CoV-2 mNG NT without pre-dilution.

8. DEVIATIONS MANAGEMENT

- All deviations encountered during assay validation will be documented in the assay validation summary report.
- Any deviation that requires testing to be stopped temporarily will include an impact statement in the summary report.

- Typographical errors are not deviations and will be corrected by the author, as required.

9. VALIDATION REPORT

A Validation Report will be written to describe the statistical analyses, the interpretation of the data, and conclusions regarding the fitness to carry out the SARS-CoV-2 mNG NT for routine serological testing.

10. REFERENCES

1. Xie X, Muruato M, Lokugamage KG et al, An Infectious cDNA Clone of SARS-CoV-2. *Cell Host & Microbe* 2020, 27 (5): 841-848.

2. Muruato AE, Fontes-Garfias CR, Ren P et al, A high-throughput neutralizing antibody assay for COVID-19 diagnosis and vaccine evaluation. *Nature Communications* 2020, in press.

3. VR-TM-10298: Manual 96-well Neutralization Assay for the Detection of Functional Antibodies to SARS-CoV-2 in Test Serum.

4. VR-SOP-LC-11299: Reagent Preparation and Vero and Vero E6 (V-E6) Thawing, Passaging, Harvesting and Plating.

5. VR-SOP-LC-11287: Preparation of Samples for SARS-CoV-2 Neutralization Assay Using the (b) (4) Workstation.

6. VR-SOP-LC-11294: Preparation of SARS-CoV-2 Stocks.

7. VR-MQR-10214: Qualification of the SARS-CoV-2 mNeonGreen Virus Microneutralization Assay.

8. VR-SOP-LC-11293: Procedure for Data Review of the SARS-CoV-2 Manual 96-well Neutralization Assay.

9. (b) (4)

11. SUPPORTIVE TABLES

11.1. Validation Run Plate Map for Dilutional Linearity Runs

Sample MDP #	Sample[a]	Sample MDP #	Sample
(b) (4)			

11.2. Validation Run Plate Map for Precision Runs

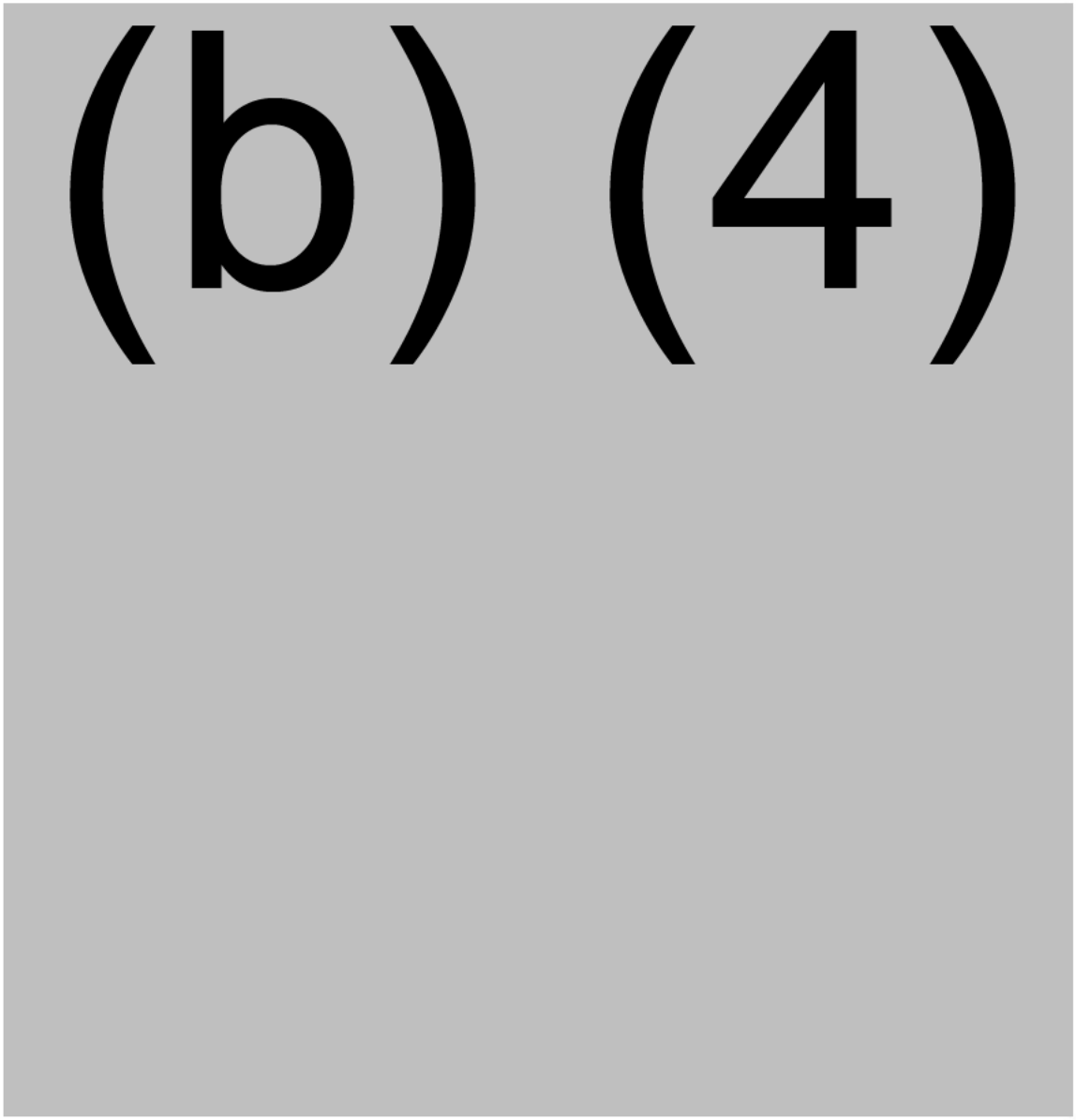
Sample MDP #	Sample[a]	Sample MDP #	Sample
(b) (4)			

11.2. Validation Run Plate Map for Precision Runs

Sample MDP #	Sample[a]	Sample MDP #	Sample
(b) (4)			

12. SUPPORTIVE FIGURES

12.1. Assay Design Schematic



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Tan, Charles	01-Dec-2020 20:06:43	Manager Approval
(b) (6)	02-Dec-2020 14:18:55	Author Approval