21120.10208 Validation Report for Establishing the Performance Characteristics of the SARS-CoV-2 S Gene Next-Generation Sequencing

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1.0	Approved and Current	Initial version	23-Jan-2021	26-Jan-2021	Indefinite

Linked Documents

- 21120.10008 Validation Protocol for Establishing the Performance Characteristics of the SARS-CoV-2 S Gene Next-Generation Sequencing

Validation Report for Establishing the Performance Characteristics of the SARS-CoV-2 Spike Gene Next-Generation Sequencing

A. Introduction/Objective

An outbreak of coronavirus disease 2019 (COVID-19) caused by the 2019 novel coronavirus (SARS-CoV-2) began in Wuhan, Hubei Province, China in December 2019, and has spread throughout China as well as numerous other countries, including the United States. The outbreak was declared a Public Health Emergency of International Concern on 30 January 2020 by the World Health Organization. Signs and symptoms of COVID-19 include fever, cough, and shortness of breath. Person-to-person spread of SARS-CoV-2 appears to occur mainly by respiratory transmission. How easily the virus is transmitted between persons is currently unclear. Based on the incubation period of illness for Middle East respiratory syndrome (MERS) and severe acute respiratory syndrome (SARS) coronaviruses, as well as observational data from reports of travel-related COVID-19, CDC estimates that symptoms of COVID-19 occur within 2–14 days after exposure. Preliminary data suggest that older adults and persons with underlying health conditions or compromised immune systems might be at greater risk for severe illness from this virus.

This assay uses conventional PCR chemistry for amplification of a SARS-CoV-2 target sequence from nucleic acids that have been extracted from nasopharyngeal swab specimens. The amplification step is followed by purification of the PCR products which is followed by visualization and concentration of the PCR products using the Agilent 2200/4200 TapeStation. Amplified samples are diluted, and libraries are prepared and indexed using the Illumina Nextera XT Library Prep Kit. Following a bead-based purification step, prepared libraries are quality checked on the Agilent 2200/4200 TapeStation. Libraries are then diluted, pooled, denatured, and loaded into a sequencing reagent cartridge for processing on the Illumina MiSeq instrument.

B. Scope

This validation report is intended to provide documented evidence of the following performance characteristics for the laboratory-developed SARS-CoV-2 S gene NGS assay using human nasopharyngeal (NP) swab specimens as defined by Validation Protocol 21120.10008 *Validation Protocol for Establishing the Performance Characteristics of the SARS-CoV-2 S Gene Next-Generation Sequencing*:

- Analytical sensitivity: limit of detection (LOD)

(b) (4)

The performance characteristics of SARS-CoV-2 S gene NGS assay includes:

- Evaluation of the isolation of nucleic acid
- Amplification by endpoint or conventional Reverse Transcriptase PCR (RT-PCR)
- Sequencing of nucleic acids by next-generation sequencing.

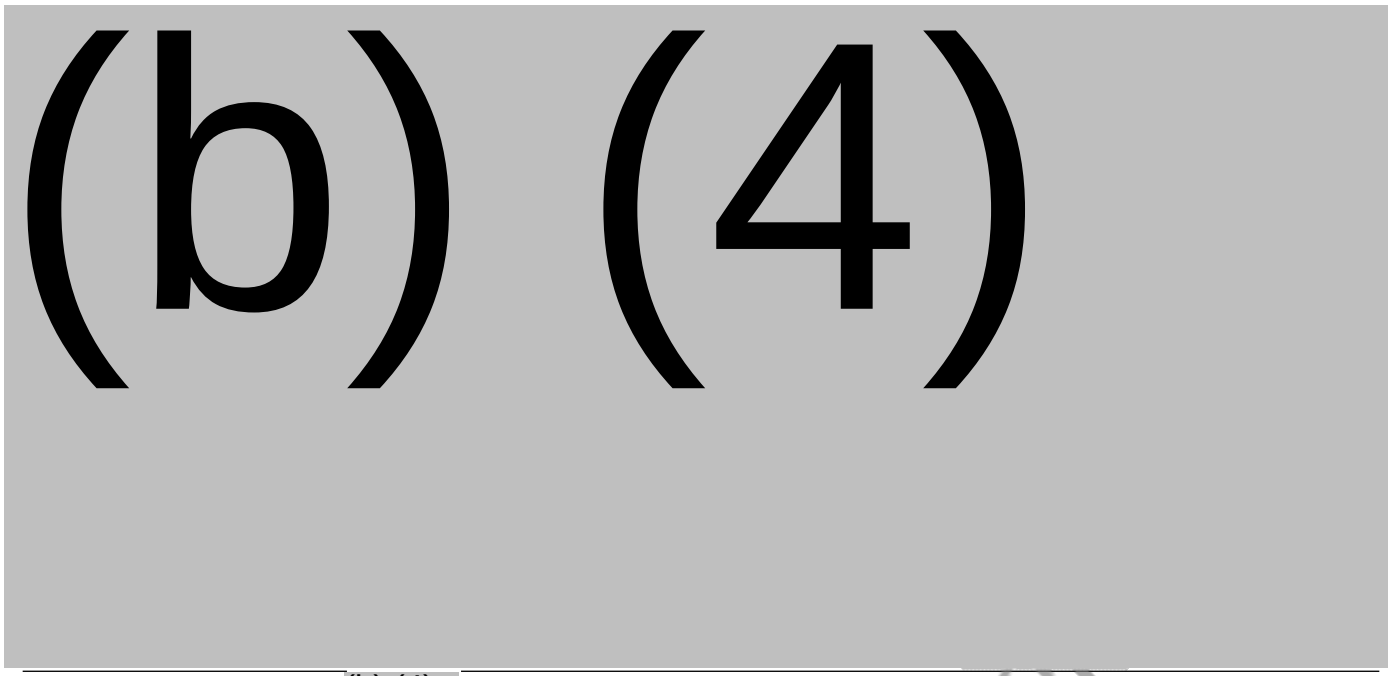
Table 1a. Summary of Validation Criteria and Results for the SARS-CoV-2 S Gene NGS Assay in NP Swabs

Performance Characteristic	Action and Acceptance Criteria	Validation Results	Pass/Fail
Analytical Sensitivity: Limit of Detection (LOD)	Action NP[1] swab specimens were (b) (4) (b) (4) (b) (4) (b) (4) with (b) (4) replicates per (b) (4)(b) (4) were prepared. Samples were taken through the entire process (extraction, RT-PCR, library preparation and sequencing analysis) unless there are no visible bands after RT-PCR analysis and or tape station readings do not meet quality criteria. Note: some replicates of the lower concentration samples were not run on the TapeStation due to lack of reagents.[2] Acceptance Criteria LOD for the SARS-CoV-2 S Gene NGS assay will be the lowest concentration detected by next-generation sequencing with (b) (4) replicates. The TapeStation readings must produce a band for S gene amplicons S1, S2, S3, and S4 to proceed with sequencing.	The lowest concentration detected with all replicates for all 4 amplicons was 6,667 copies/mL. Sequencing acceptance criteria was determined to be (b) (4)	As found
(b) (4)			Pass
			Pass

Table 1a. Summary of Validation Criteria and Results for the SARS-CoV-2 S Gene NGS Assay in NP Swabs			
Performance Characteristic	**Action and Acceptance Criteria**	**Validation Results**	**Pass/Fail**
	(b) (4)		

[1] Nasal swab specimens were used for validation purposes, though the sample type for analysis is nasopharyngeal swabs. See explanation in Procedures section, below.
[2] See deviation 2, Section H.

Table 1b. Stability characteristics to be established for the SARS-CoV-2 S Gene NGS Assay			
Performance Characteristic	**Action**	**Stability Criteria**	**Stability Results**
(b) (4)			



[1] It is recommended not to (b) (4) these samples. Data acquired with artificially created samples did not support (b) (4) This finding is consistent with previous qPCR testing.

C. Abbreviations and Definitions

Table 3. Abbreviations and Definitions Used in this Document

Acronym/Word/Temperature Range to be Defined	Definition
-80°C	-64°C to -90°C
-20°C	-15°C to -35°C
Refrigeration (4°C)	2°C to 8°C
Ambient/Room Temperature	15°C to 25°C
37°C	36°C to 38°C
BSC	Biosafety Cabinet
°C	Degrees Celsius
Cps/mL	Copies per milliliter
Cps/Rxn	Copies per reaction
(b) (4)	
DF	Dilution Factor
DNA	Deoxyribonucleic Acid
FDA	Food and Drug Administration
IVT	*In vitro* Transcript
LOD	Limit of Detection
(b) (4)	
mL	milliliter
N/A	Not Applicable
ND	Not Detected
(b) (4)	
NP	Nasopharyngeal Swabs
NS	Nasal Swabs
(b) (4)	
PEC	Positive Extraction Control
qPCR	Quantita ive Real-Time PCR
(b) (4)	
RNA	Ribonucleic Acid
S	Spike gene
SD	Standard Deviation
SOP	Standard Operating Procedure
(b) (4)	
TBD	To Be Determined
µg	microgram
(b) (4)	
µL	microliter
(b) (4)	
UTM	Universal Transport Medium
VTM	Viral Transport Medium
(b) (4)	

D. Key Personnel

Table 2. Key Personnel			
Initials	**Name**	**Title**	**Role**
(b) (6)			
TJM	Tyler Moss	Senior Data Analyst, Oncology Diagnostics	Data Review
(b) (6)			

E. Materials

The following materials (or suitable equivalents) will be used:

Table 4. Reagents and Materials			
Description	**Source**	**Lot #**	**Expiration Date**
(b) (4)			
SARS-CoV-2 Positive Sample	Viracor Eurofins	(b) (4)	
Extraction			
EasyMAG Lysis Buffer	BioMerieux	Z012CG1LB Z012EM1LB Z012ER1LB	28-Mar-2022 28-May-2022 28-May-2022
EasyMAG Extraction Buffer 1	BioMerieux	Z012DE Z012FH Z012CU Z012FO Z012EP	31-Apr-2022 30-Jun-2022 31-Mar-2022 30-Jun-2022 31-May-2022
EasyMAG Extraction Buffer 2	BioMerieux	Z011MG Z011HD Z011KL Z011MU	30-Nov-2021 31-Aug-2021 30-Sep-2021 30-Jun-2022
EasyMAG Extraction Buffer 3	BioMerieux	Z011EH Z011GN Z011GK Z011GR	31-May-2021 31-Jul-2021 31-Jul-2021 31-Jul-2021
Magnetic Silica	BioMerieux	Z011DQ1MS Z011DO1MS Z011BQ1MS	21-Dec-2020 28-Apr-2021 25-Jan-2021
Amplification			
SARS-CoV-2 S Gene Conventional RT-PCR primer mixes	(b) (4)	See below	
(b) (4)			

Table 4. Reagents and Materials			
Description	**Source**	**Lot #**	**Expiration Date**
Qiagen QIAquick One-Step RT-PCR Kit	Qiagen	166025932 166018860	08-Jun-2021 30-Mar-2021
5x Qiagen buffer	Qiagen	16624396 166014751	08-Jun-2021 30-Mar-2021
Qiagen Enzyme Mix	Qiagen	166014687 166018247	08-Jun-2021 30-Mar-2021
dNTP Mix	Qiagen	16609182 166016983	08-Jun-2021 30-Mar-2021
(b) (4)			
D5000 ScreenTape	Agilent	0201893-34 0201924-108 0201924-105 0201924-85 201924-151	07-Apr-2021 01-Jun-2021 01-Jun-2021 01-Jun-2021 01-Jun-2021
D5000 Reagents	Agilent	0006545287 0006550928	30-Jul-2021 30-Nov-2021
(b) (4)			
Library Preparation			
Illumina Nextera XT Library Prep Kit	Illumina	See below	
Tagment DNA Buffer (TD)	Illumina	20455987 20491563	28-May-2021 31-Oct-2021
Amplicon Tagment Mix (ATM)	Illumina	20435366 20436468	14-Feb-2022 01-Apr-2022
Neutralize Tagment Buffer (NT)	Illumina	20473725 20443725	02-Apr-2022 02-Apr-2022
Nextera PCR Master Mix (NPM)	Illumina	20463302 20489201	24-Jun-2021 28-Oct-2021
Nextera XT Index Primers	Illumina	20480201	23-Feb-2022
Illumina Nextera Index Kit	Illumina	See below	
Resuspension Buffer (RSB)	Illumina	20464550 20488713	22-Jul-2021 26-Oct-2021
(b) (4)			
Library Sequencing			
(b) (4)			
(b) (4)			
Assay Controls			
SARS-CoV-2 Whole Virus Positive Control	(b) (4)		
(b) (4)			

Table 4. Reagents and Materials			
Description	**Source**	**Lot #**	**Expiration Date**
(b) (4) nuclease free H2O	(b) (4)		
(b) (4)	(b) (4)		

Table 5. Equipment and Supplies	
Description	**Instrument ID**
Refrigerator capable of sustaining 2-8°C	
Freezer capable of sustaining -15°C to -35°C	
Freezer capable of sustaining -64°C to -90°C	
Biological Safety Cabinet, Class II	(b) (4)
Fixed-angle benchtop centrifuge (with rotor for 2mL tubes)	
(b) (4) swing-bucket centrifuge	
BioMerieux easyMAG	
(b) (4) Thermal Cycler	
(b) (4) Sequencer	
Agilent 2200 or 4200 TapeStation	
(b) (4) 96-well MIDI plate magnetic stand	
(b) (4) vortexer	
PCR strip tube mini-centrifuge	
(b) (4) High-speed micro-plate shaker	(b) (4)
(b) (4) PCR tubes or (b) (4) Optical 96-well Reaction Plate (standard plate)	
(b) (4) caps or Optical Adhesive Covers	
2mL non-skirted (b) (4) tubes	
(b) (4) Optical Tube Strips (8x)	
(b) (4) Optical Tube Strip Caps (8x)	
96-well conical-bottom MIDI plate	

Table 6. SARS-CoV-2 S Gene Sequencing Assay Primer Sequences		
Oligonucleotide	**Oligonucleotide sequence 5' → 3'**	**Final Concentration nM**
(b) (4)		

F. Procedures

General Methods

Nasal Swab Specimen Preparation

Note: Nasopharyngeal swabs were not available during the validation testing; nasal swabs were used as a substitute. Nasopharyngeal swabs are typically collected by trained healthcare personnel or self-collection is monitored by a supervisor (not available onsite at the time of collection). Nylon flocked swabs were used in this study. In general, nylon flocked swabs are routinely used for nasopharyngeal sampling.

DNA Quality Check by Agilent TapeStation

The Agilent 2200 or 4200 TapeStation in conjunction with D5000 ScreenTapes, D5000 reagents, and the TapeStation Analysis software was used to assess post-amplified and purified PCR reactions for the presence, size, and concentration of any products generated. The values reported in the Sample Table (i.e. total DNA concentration in the sample) was used to determine dilution factors for subsequent library preparation. A portable digital file (.pdf) along with a Sample Table comma separated value (.csv) file were generated for each run performed. The Sample Table was formatted using Excel and printed for inclusion in the run packet.

Library Quality Check by Agilent TapeStation

The Agilent 2200 or 4200 TapeStation in conjunction with D5000 ScreenTapes, D5000 reagents and the TapeStation Analysis software was used to assess purified libraries for presence, average fragment size, and concentration of the fragment distributions generated. The (b) (4) (b) (4) functions of the software was used to (b) (4) (b) (4) in order to generate the metrics described above. A portable digital file (.pdf) along with a Region Table comma separated value (.csv) file was generated for each run performed. The Region Table was formatted using Excel and printed for inclusion in the run packet. The values reported in the Region Table were used to determine dilution factors for subsequent library normalization.

Sequencing Analysis

Analysis of next generation sequencing data for the SARS-CoV-2 S gene NGS assay was done using Qiagen CLC Genomics Workbench v20.0.1 using NC_045512.2 as the reference strain. The custom workflow in CLC Genomics Workbench processed the sequencing data as follows: paired fastq files were imported, primer sequences were trimmed from 5'-ends of reads, reads were mapped to the full SARS-CoV-2 reference genome (NC_045512.2), single nucleotide and insertion/deletion variants relative to reference were called and annotated, and a consensus sequence of the spike gene (bases 21615 to 25436) was generated. The analysis workflow reported annotated variant tables, spike-gene coverage tables, and spike-gene consensus sequences.

Acceptance criteria for controls and negative samples

Upon TapeStation D5000 assessment and subsequent analysis of data using the TapeStation Analysis software: if the viral load was insufficient to obtain a correct band for the SARS-CoV-2 S gene targets (S1 (1026bp), S2 (893bp), S3 (1178), and S4 (1264bp), these results were considered negative. (b) (4) This validation served to determine the (b) (4)

(b) (4)

Acceptance criteria for RT-PCR amplicon quality

Positive results for the RT-PCR reactions were identified by 1) the presence of a band at the appropriate size for the SARS-CoV-2 S gene PCR products (S1 (1026bp), S2 (893bp), S3 (1178), and S4 (1264bp) relative to the D5000 ladder and 2) a peak table reporting a concentration for the

specific bands for the sample in question. The presence of secondary products was accounted for prior to moving into library preparation by using the concentration reported in the Sample Table to determine dilution factors used.

Acceptance criteria for Sequencing

Sequencing data was assessed using both global run metrics provided by the MiSeq upon run completion as well as individual library sequence quality metrics. Guidelines from Illumina were referenced to determine run metrics thresholds. SARS-CoV-2 S Gene NGS runs using MiSeq v2 chemistry reagents running paired-end 2 x 151 reads must exhibit:

- Cluster densities approximating 600 – 1,200K/mm^2
- > 80% of bases called exhibit Q-scores ≥30

Individual library sequence quality metrics were assessed by referencing the sequencing quality reports generated by analysis through the Qiagen CLC workbench program. For SARS-CoV-2 S gene NGS runs, up to 24 libraries can be sequenced on a single flow cell and the number of reads displayed in the trim summary section of the trim report should be ≥ 50,000 reads for each amplicon (prior to the reads being trimmed). Nucleotide positions between 21615-25436 should have a coverage of ≥ 100 as summarized in the mapping coverage table.

Read acceptance criteria

- Percent of reads greater than Q30 for Read 1 ≥ 80%
- Percent of reads greater than Q30 for Read 2 ≥ 80%
- 95% of spike-gene bases > 100x coverage

Performance Characteristics Evaluation

Analytical Sensitivity: Limit of Detection (LOD)

Experimental Design:

For the evaluation of analytical sensitivity (limit of detection (LOD)) of the SARS-COV-2 NGS assay, human SARS-CoV-2 qPCRnegative NS samples were (b) (4) (b) (4) through NGS. All samples were subjected to extraction, amplification, and sequencing as described above.

During the initial testing for Sensitivity, there was a deviation due to differences in how technicians were running the samples; see Deviation 1, Section H.

In addition, not all levels in the Sensitivity assay were assayed on the TapeStation due to the vendor being out of stock; see Deviation 2, Section H.

Finally, there was an anomalous reading in the (b) (4) copies/mL level; the S3 amplicon failed to amplify, resulting in (b) (4) replicates passing acceptance criteria; see Deviation 3, Section H. This level had (b) (4) replicates pass acceptance criteria in pre-validation (see data in Appendix A). To assure that the reading obtained during validation was an anomaly, the (b) (4) copies/mL sample will be (b) (4) and re-assayed. This data will be reported in an amendment to this report.

Analysis:

The sequencing results of all samples were analyzed to identify depth of coverage and call variants for each.

Acceptance Criteria:

- LOD for the SARS-CoV-2 S Gene NGS assay will be the lowest concentration detected by next-generation sequencing with (b) (4) replicates detected. The TapeStation readings must produce a band for S gene amplicons to proceed with sequencing.

(b) (4)

(b) (4)

Experimental Design:

(b) (4)

Analysis:

(b) (4)

Acceptance Criteria:

(b) (4)

(b) (4)

(b) (4)

Analysis:

(b) (4)

Acceptance Criteria:

(b) (4)

(b) (4)

Experimental Design:

(b) (4)

(b) (4)

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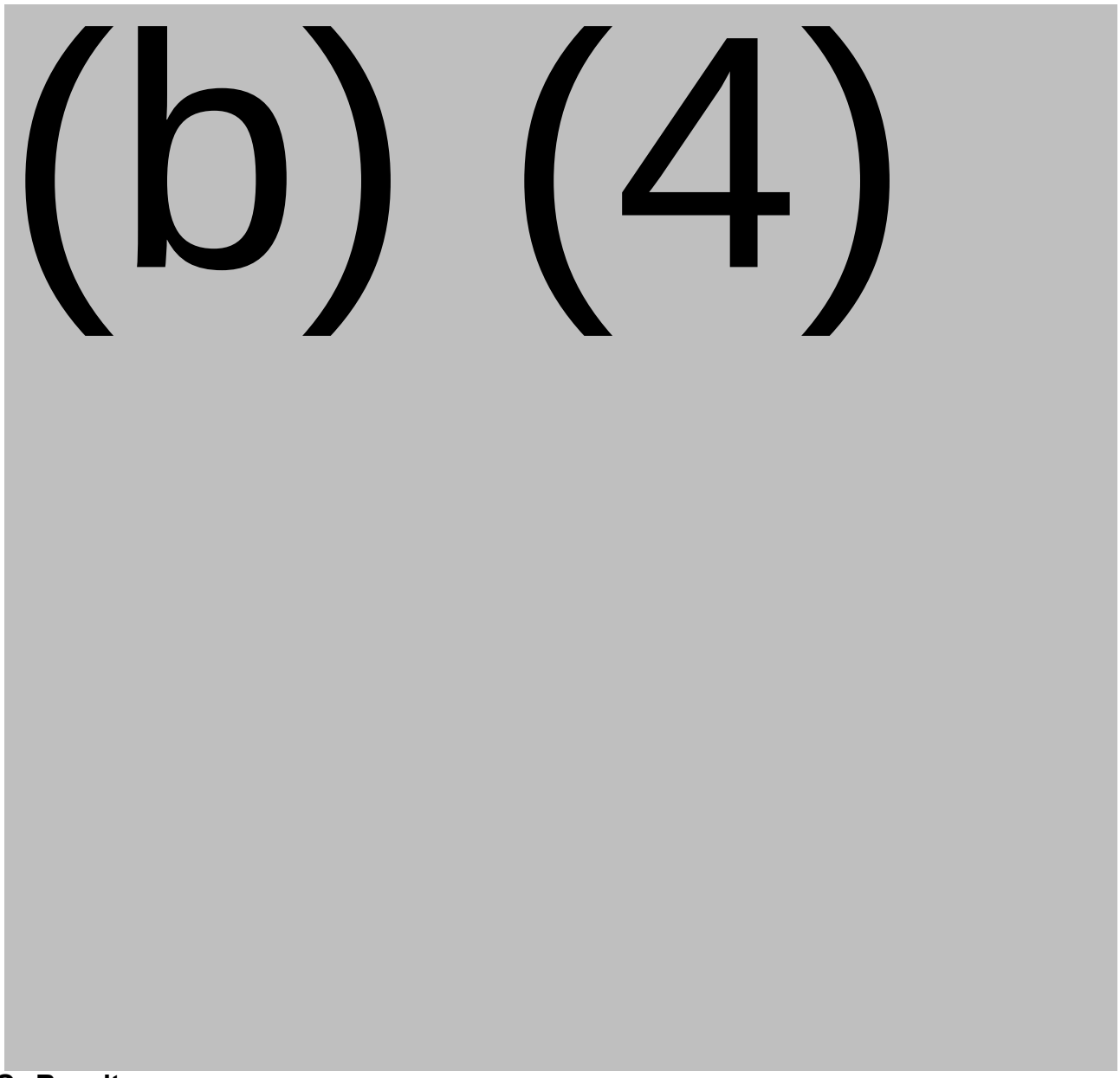

G. Results

Graphical and tabular presentations of the results are below. Please see Table 1 for textual descriptions of results. Please see Methods and Analysis sections for information pertaining to these topics. Detailed methods and results may be found in Binder **BP-2020-098**.

Analytical Sensitivity: Limit of Detection (LOD)

The limit of detection was determined to be 6,667 copies/mL (**Tables 8 and 9**).

Table 8. Results from Sensitivity Sequencing

(b) (4)

[1] See Deviation 3, Section H.

Table 9. Results from Sensitivity Amplification

(b) (4)

Table 9. Results from Sensitivity Amplification

(b) (4)

Table 9. Results from Sensitivity Amplification

(b) (4)

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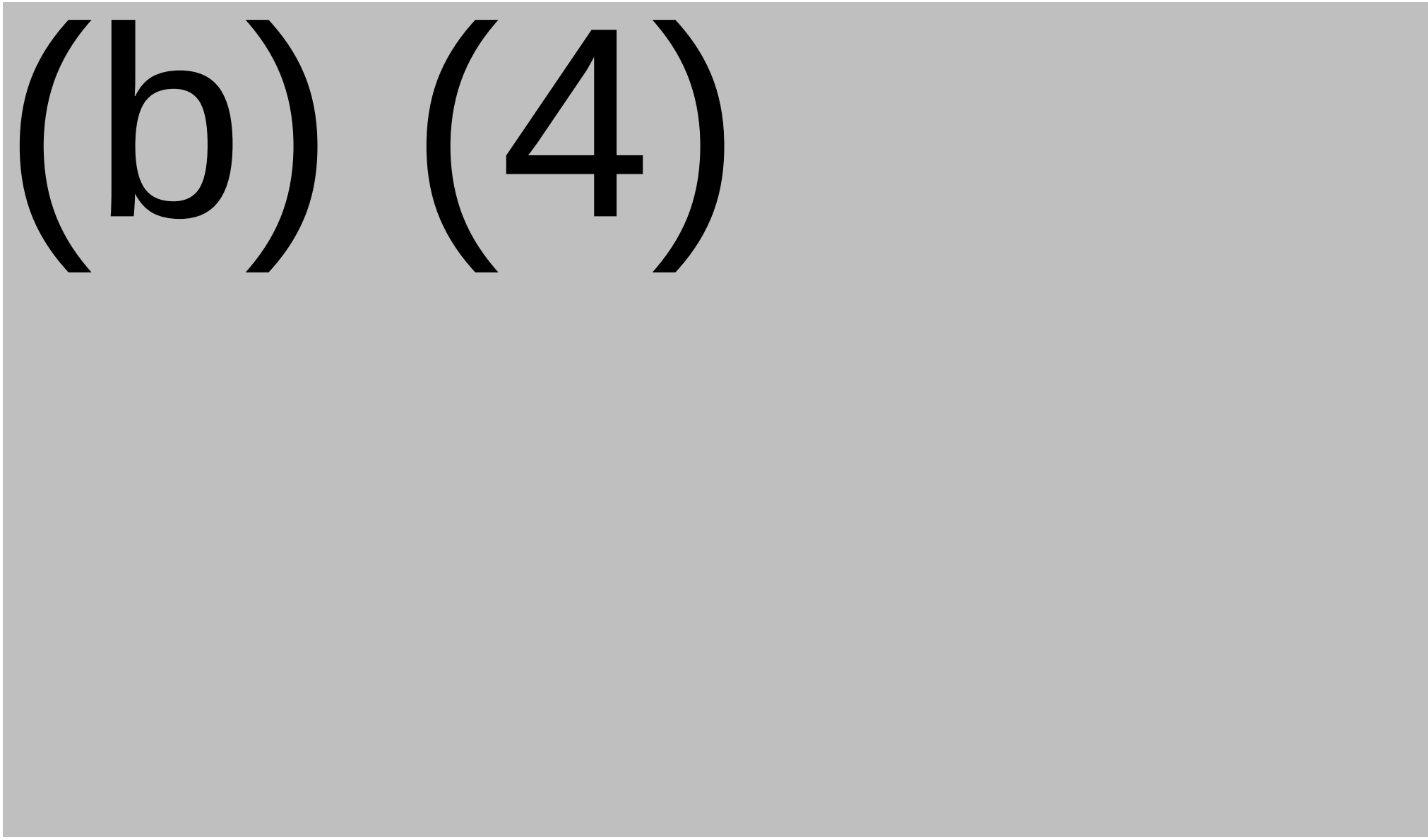

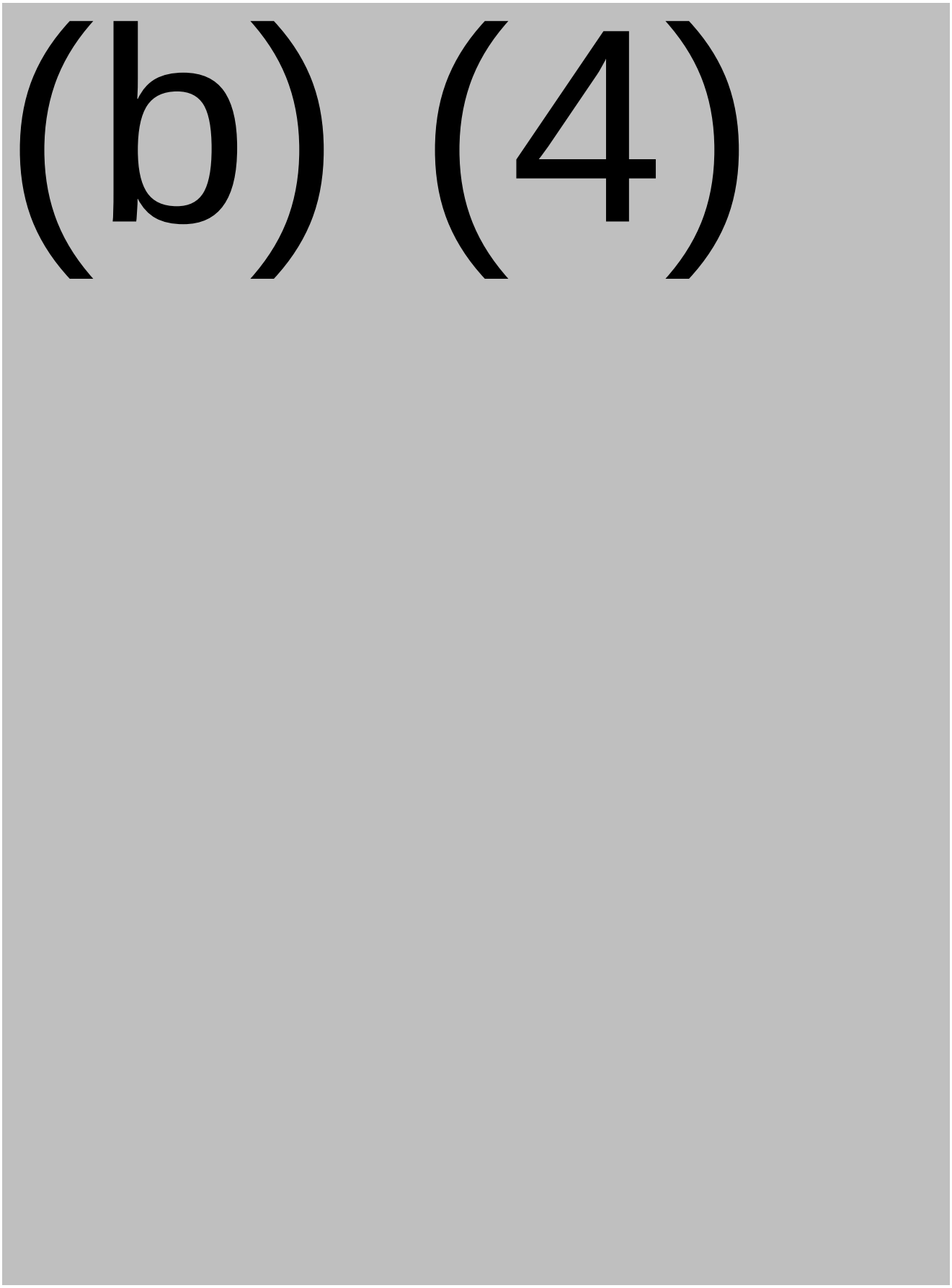

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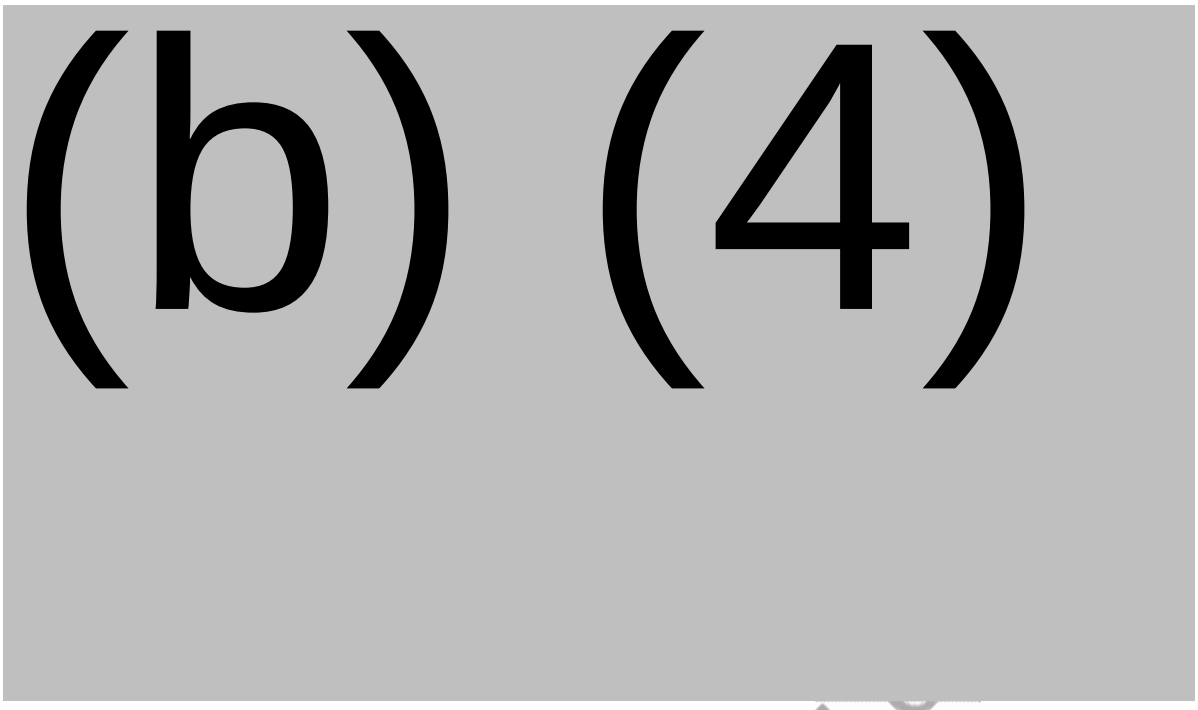

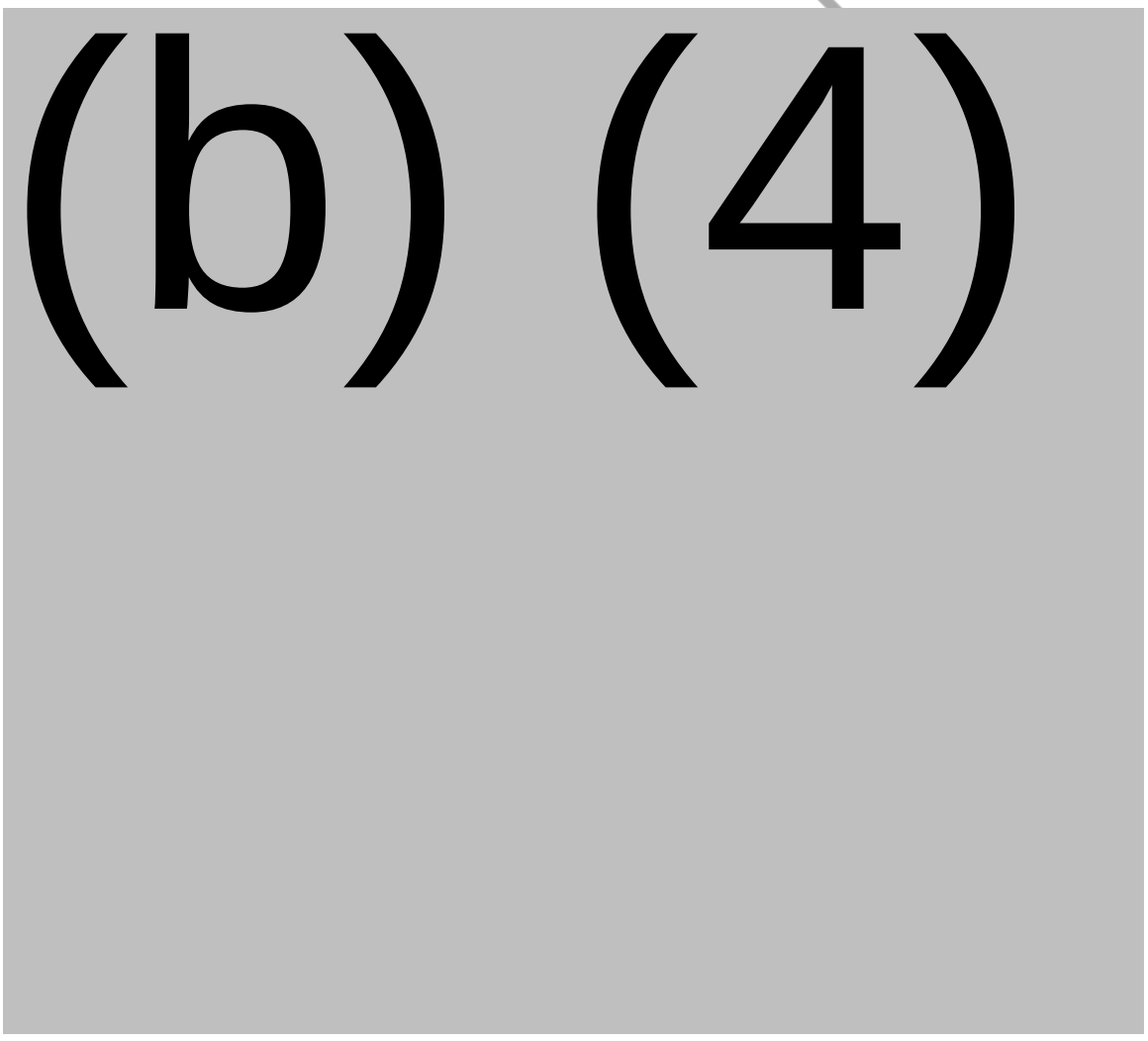

(b) (4)

(b)

Fate of Runs

All assays performed to support this validation protocol are listed below, along with the disposition of the data for each run.

Table 18. Fate of Runs

Run Date	Run Name	Purpose	Results
(b) (4)			

[1] See Deviation 1, Section H.

H. Deviations

1. Deviation 1, Sensitivity, CA-02699. In initial testing for the sensitivity portion of the protocol, there were differences between technicians in how the method was run. The analysts were re-trained on the method, and the sensitivity experiment was repeated. The data reported here was from the repeated assays.

2. Deviation 2, Sensitivity, CA-02700. Some samples tested for the sensitivity at the lower concentrations were not run on the Tapestation due to the lack of tapes. The vendor is currently out of stock, so some samples at the lower concentration were not assayed on the TapeStation. This lack of testing should not affect the conclusions drawn from this study, as the replicates run at these lower concentrations did not generate bands in TapeStation testing. Not testing other replicates at the same concentration would not affect the conclusions drawn from the data.

3. Deviation 3, Sensitivity. The testing established that 6,667 copies/mL met the acceptance criteria set for this assay. However, there was an anomalous result observed at (b) (4) copies/mL; S3 failed to amplify. An additional (b) (4) copies/mL sample was extracted and used for amplification replating. The replating amplification produced an S3 amplicon at (b) (4) and was processed through NGS library preparation with S1, S2, and S4 amplicons from the first attempt. Sequencing data indicated coverage drop-out in the S3 amplicon region for the replating attempt. Because the 10,000 copies/mL level also met the acceptance criteria, the LOD was set at the lowest level at with all replicates detecting all amplicons, which was 6,667 copies/mL.

   It was thought that the lack of detection of the missing amplicon was due to the low concentration of the amplicon (b) (4) which is typically below the threshold where a sample will advance to the sequencing step. The (b) (4) copies/mL concentration did have 4 out of 4 amplicons detected for (b) (4) during the pre-validation sensitivity assessment (See Appendix A for data), which also points to the lack of detection of the S3 amplicon in the one replicate as an anomaly. To address this anomaly, an additional study will be implemented that repeats the (b) (4) copies/mL sample to ensure that 4 out of 4 amplicons are detected at that concentration. That data will be reported in an amendment to this report once the testing is completed.

I. Conclusions

The enclosed data shows that the SARS-CoV-2 NGS assays are (b) (4) for NP swabs as a matrix. The data also shows the limit of detection.

(b) (4)

Based on the entirety of the results from the qualification study, the performance characteristics of the SARS-CoV-2 Next Generation Sequencing assay met the acceptance criteria specified in 21120. 10008 *Validation Protocol for Establishing the Performance Characteristics of the SARS-CoV-2 S Gene Next-Generation Sequencing*. Therefore, human NP swab specimens will be accepted for testing in the SARS-CoV-2 NGS assay.

J. Related Documents

Validation Protocol 21120.10008 *Validation Protocol for Establishing the Performance Characteristics of the SARS-CoV-2 S Gene Next-Generation Sequencing*

SOP 21120.705 *NucliSens easyMAG & eMAG Total Nucleic Acid Extraction*

SOP 21120.5983 *Agilent Tapestation Operation, Maintenance, and Calibration*

SOP 21120.7375 *Illumina MiSeq Operation, Maintenance, and Calibration*

21120.443 *Thermal Cycler Operation, Maintenance, and Calibration of the* (b) (4) *Thermal Cycler*

SOP 21120.269 *Records Management*

SOP 21120.9806 *NGS Data Retention*

K. References

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Appendix A

Pre-Validation Data for Assay Sensitivity: Limit of Detection

Pre-validation testing used (b) (4) (b) (4) listed in the table below. Results of this testing show that all 4 amplicons were detected for (b) (4) replicates tested at the (b) (4) copies/mL concentration.

Table 19. Pre-Validation Sensitivity Amplification

(b) (4)

Table 19. Pre-Validation Sensitivity Amplification

(b) (4)

Table 19. Pre-Validation Sensitivity Amplification

(b) (4)