High Throughput SHERLOCK CRISPR SARS-CoV-2 Test

Abstract:

The ability to control the spread of COVID-19 has been hampered by a lack of rapid, scalable, and easily deployable diagnostic solutions. Efforts to increase testing capacity have been adversely impacted by supply chain challenges due to dependencies on a limited set of reagents, consumables, and instrumentation. Here, we present a diagnostic method based on CRISPR (clustered regularly interspaced short palindromic repeats) that can deliver sensitive and specific detection of SARS-CoV-2, with the potential for up to 5,000 patient samples per day without thermal cycling instrumentation, and with minimal operator hands-on time. The assay utilizes SHERLOCK (Specific High-sensitivity Enzymatic Reporter unLOCKing) for the qualitative detection of SARS-CoV-2 RNA and may be performed directly on a specimen with minimal sample treatment. The assay is implemented in a 384-well format that is compatible with automated liquid handling instrumentation and provides results in less than one hour. Assay performance was evaluated with 105 (60 negative, 45 positive) SARS-CoV-2 specimens tested using FDA emergency use authorized assays (Hologic Panther, Roche Cobas and Perkin Elmer). The high throughput SHERLOCK SARS-CoV-2 assay was 100% concordant with the reference methods, correctly detecting all positive and negative samples.

Introduction:

A significant increase in available SARS-CoV-2 testing has been recognized as a critical requirement to end the COVID pandemic [1]. Most current tests rely on PCR-based amplification and detection of viral RNA, and require expensive, complex and sensitive equipment with highly trained laboratory personnel to operate it [2]. As such, the ability to quickly scale up the volume of testing required to meet demand has been challenging and, in many cases, leads to large delays in results being returned to the patient [3]. Isothermal amplification of viral targets has greatly reduced the complexity of equipment required to amplify viral targets, however off target amplification leading to false positives is a problem when using these methods alone [4–9]. Methods combining the flexibility and simplicity of an isothermal amplification with a high level of specificity are needed.

In recent years, CRISPR-based diagnostics have emerged as a programmable method for rapid, sensitive, and specific detection of nucleic acids [10–12]. CRISPR-based diagnostics utilize the specific recognition of a target nucleic acid sequence by a guide RNA/Cas protein complex, which activates collateral nuclease activity of the Cas12 or Cas13 protein complex [12–16]. This collateral activity can be converted into various readouts, including lateral flow or fluorescence. Utilizing a highly active Cas13a protein from *L. wadei* (LwaCas13a) combined with isothermal amplification, the SHERLOCK (Specific High-sensitivity Enzymatic Reporter unLOCKing) platform was developed as a low-cost CRISPR-based diagnostic that enables detection of DNA or RNA with single-nucleotide specificity [15,16]. We have further enhanced the robustness and performance of this method by incorporating a highly sensitive LAMP-based amplification of the target viral RNA.

In May of this year, FDA issued the first Emergency Use Authorization (EUA) for a CRISPR diagnostic test when it granted an EUA for the SHERLOCK CRISPR SARS-CoV-2 kit. The SHERLOCK CRISPR SARS-CoV-2 kit (SHERLOCK kit) is capable of detecting the presence of

a target nucleic acid in approximately 1 hour with a Limit of Detection (LoD) of 6.75 copies per microliter of VTM (viral transport medium)[17]. The SHERLOCK assay was authorized for the detection of SARS-CoV-2 nucleic acid in upper respiratory tissue samples including nasal swabs, nasopharyngeal swabs, oropharyngeal swabs, nasopharyngeal wash/aspirate or nasal aspirate and bronchoalveolar lavage specimens collected from individuals suspected of COVID-19 by their healthcare provider. It was recently reported in an independent clinical evaluation that the SHERLOCK assay was 100% concordant to RT-PCR in the detection of SARS-CoV-2 in clinical nasopharyngeal samples [18].

Loop-mediated isothermal amplification (LAMP) has been extensively studied as a molecular diagnostic amplification method for various viruses including SARS-CoV2 [19,20]. LAMP based amplification methods are attractive for diagnostics because LAMP has been reported to be more tolerant than PCR to endogenous inhibitors present in biological samples [21,22]. However, traditional LAMP-based detection methods suffer from poor specificity and are challenging to multiplex [5–9]. Our SHERLOCK-based methods overcome this limitation with sequence specific Cas13 based amplicon detection.

Here we present several advancements that were developed to improve the simplicity and throughput of the CRISPR diagnostic method implemented in the SHERLOCK kit. These include combining two independent SARS-CoV-2 targets, Nucleocapsid (N) and Open Reading Frame (ORF), in a single reaction, simplifying the sample preparation, and implementing the assay in a 384-well format with minimal liquid handling steps to increase throughput and improve compatibility with automated processes. Additionally, we evaluated multiple extraction methods and demonstrated that a simple heat and proteinase K treatment is sufficient to allow direct sample (swab in saline, saliva) addition to a SHERLOCK reaction while maintaining high sensitivity (2 copies/uL) and specificity.

Results:

SHERLOCK High-throughput Method with 96-well RNA Extraction

Here, we developed a workflow based on the SHERLOCK CRISPR SARS-CoV-2 test that increases throughput, simplifies sample preparation, and combines dual target SARS-CoV-2 amplification and detection into a single reaction (Figure 1). Extracted gRNA samples are added to a LAMP reaction master mix, with primers specific to the target, in a 384-well deep well, fluorescence-compatible plate. The LAMP reactions are then topped with 20 µl of molecular-biology grade mineral oil to prevent condensation and reduce the risk of contamination. The LAMP reaction occurs on any plate heater capable of maintaining 61ºC. The time of the LAMP reaction depends on the template, and is approximately 30 minutes for samples extracted with a nucleic acid extraction kit. After allowing the plate to cool to room temperature, the LAMP reaction plate is moved to a dead air/ post-amplification area and the plate seal is removed. This plate seal can either be disposable or a silicone seal compatible with automated plate handlers. The Cas detection mix (10 µl) containing the Cas enzyme as well as the guide RNA specific for the amplified target is added to each well. The plate is then read on a fluorescence plate reader over 10 minutes at 37ºC. With this method a single operator can process 190 samples in 70 minutes (excluding extraction).

To demonstrate the robustness of the SHERLOCK High Throughput method, we evaluated several bead-based RNA extraction kits on pooled negative NP swab matrix spiked with Zeptometrix NATtrol SARS-CoV-2 viral particles at decreasing concentrations. We tested three

commercially available kits: MagMAX Viral/Pathogen Isolation kit, MagMAX Viral RNA isolation kit and the Zymo Quick-DNA/RNA Viral MegBead kit (Supp Table 2A). All three kits had comparable LoDs, however the workflow of the MagMAX Viral RNA was most conducive to our manual protocol and we tested additional concentrations. With this workflow, our LoD was 2cp/µl (20/20 ,100%) (Fig 2A). We also compared the sensitivity of three fluorescent plate readers (Biotek Neo2, Tecan MPlex and Fluoroskan Microplate Fluorometer) to expand accessibility of our assay. All three plate readers had similar LoD of viral genomic RNA spiked into the reaction (4cp/µl for the Tecan, 2cp/µl for the Biotek and Fluoroskan instruments) (Supplemental Table 2B). To determine cross reactivity and competitive inhibition, we tested the high-throughput workflow with a panel of genetically related organisms as well as common high-priority organisms circulating in the area. Data shown in Supplemental Table 2C, D shows 100% specificity towards SARS-CoV-2. To test our clinical sensitivity, we tested 30 positive and 30 negative NP samples, previously tested by molecular diagnostic methods in CLIA labs and confirmed in house using a modified CDC EUA protocol (Supplemental Table 2E), extracted using the MagMAX Viral RNA isolation kit, with a 30-minute LAMP reaction. Our clinical evaluation resulted in a PPA (Positive predicted agreement) of 100% (30/30) and a NPA (Negative predicted agreement) of 100% (30/30) for the SHERLOCK high throughput workflow (Figure 2 B-D). These data support that the SHERLOCK high-throughput workflow for the detection of SARS-CoV-2 in upper respiratory specimens is sensitive, specific and improves the overall turnaround time as compared to Sherlock's EUA kit.

SHERLOCK High-throughput Direct method

In addition to increasing the throughput of our assay, we were interested in developing a workflow that allowed for use of samples that have not gone through a full RNA extraction process, as there have been consistent shortages of RNA extraction materials [23]. The SHERLOCK Direct workflow begins with a simplified sample treatment where 2 µl of proteinase K is added to 18 µl of each sample. The samples are then heated for 6 minutes at 65ºC followed by 98ºC for 3 minutes to heat-kill the proteinase K enzyme, followed by cooling to 4-10ºC. These samples can be heated in a 96 or 384-well PCR plate on a heat block. Samples can then be added directly to the LAMP reaction as detailed above, with the amplification time extended to 40 minutes.

With an automated implementation parallel processing four 384 well plates, this SHERLOCK Direct method can process 9216 samples per day (Supplemental Table 1A,B).

Saline has become a commonly used storage solution for nasal swabs [24] with high levels of accuracy and stability. We tested the compatibility of our high-throughput workflow with NP swabs in saline with the SHERLOCK Direct workflow. First, we established the LoD of our workflow with saline spiked with SARS-CoV-2 inactivated particles from 100cp/ µl to 0.01 cp/µl. Our LoD for contrived saline samples was 10cp/µl (22/23, 95.7%) (Figure 3A). We then tested the clinical applicability of our SHERLOCK Direct method on clinically collected NP swabs stored in 0.9% saline, 20 positive and 25 negative samples. All samples were purchased from a biobank after having been tested and confirmed COVID positive or negative by an outside CLIA lab. To ensure sample integrity, we also tested the material using an in-house developed protocol modeled on the CDC EUA protocol, i.e., extraction using the Qiagen RNA kit and RT-PCR using the CDC primers targeting the N gene of SARS-CoV-2 and the internal control of RnaseP (Supplemental table 3A). The PPA for the SHERLOCK Direct high throughput method in saline was 100% (25/25) and the NPA was 100% (20/20, Figure 3 B-D), while reducing the

time to result and cost associated with sample extraction. We also tested saliva with our SHERLOCK Direct method. We were able to show high analytical sensitivity, 5cp/µl in pooled saliva (Supplemental Table 3B).

Discussion:

Most of the population will not have access to a COVID-19 vaccine for many months, therefore testing remains crucial for controlling the spread of the virus [25]. The most recent report on average time to results issued by The COVID States Project is 2.7 days with 42% of people waiting at least 3 days [3], which is too long for reliable contact tracing. Critical to increasing testing capacity is high throughput molecular testing that is not affected by supply chain limitations. Here we demonstrated a high-throughput method for detecting SARS-CoV2 down to 2 copies/µL using a high throughput magnetic bead-based purification of patient samples, or 10 copies/µL direct from patient samples. We also verified key improvements to the SHERLOCK CRISPR SARS-CoV-2 EUA protocol that resulted in an increased number of samples processed and a decreased time to result. With this high throughput method, 96 samples can be tested manually in 100 minutes.

Overall, the improvements demonstrated here are: i) a simplified workflow with decreased liquid handling steps, ii) transition to a 384 well plate format beginning at the sample prep step or LAMP step, and iii) removal of the need for RNA purification of patient samples. Additionally, we demonstrated that this method is compatible with multiple plate readers and magnetic bead RNA isolation kits. Most importantly this high-throughput SHERLOCK CRISPR SARS-CoV-2 test shows 100% specificity and 100% sensitivity.

The modifications to the SHERLOCK CRISPR SARS-CoV-2 EUA protocol demonstrated here result in the development of a more user friendly, faster, inexpensive and robust method for detecting SARS-CoV-2 direct from patient samples. This test can be run on common lab equipment different from what is used for RT-qPCR assays, allowing for individual labs that are currently using COVID-19 RT-qPCR tests to increase their testing capacities. Implementation of this test can increase testing capacity that may enable more efficient and reliable contact tracing and decrease the spread of COVID-19.

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Figures:

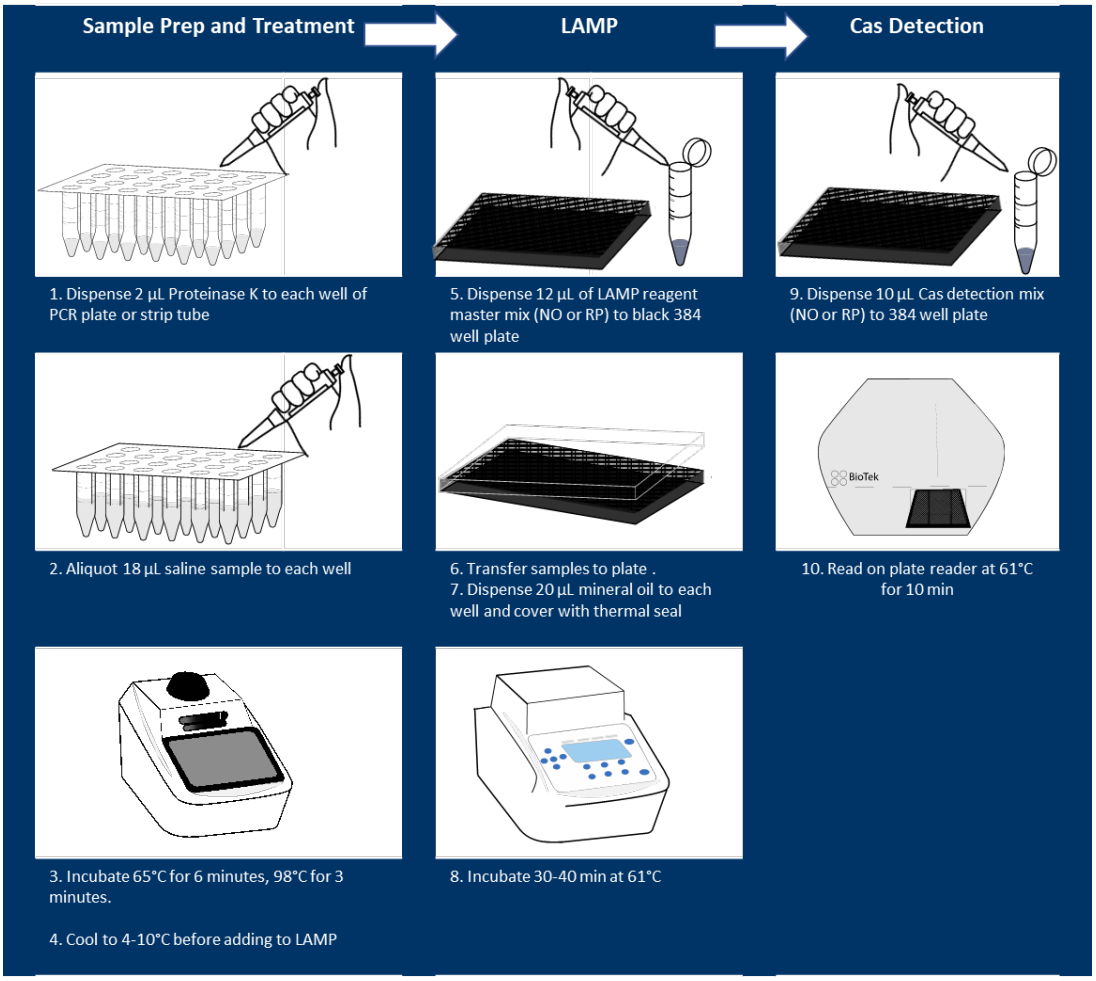
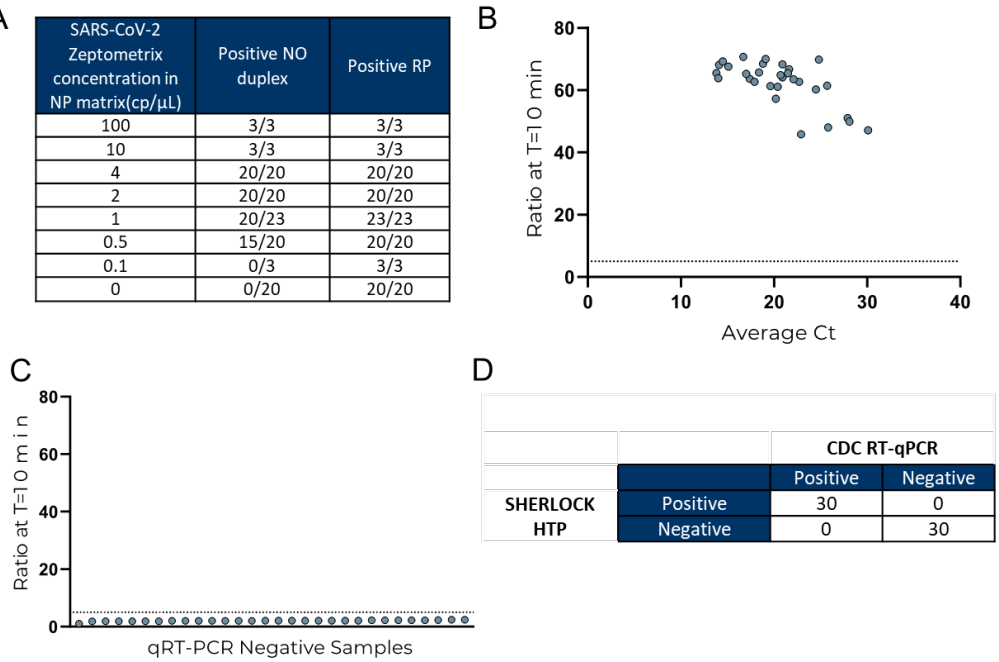


Figure 1. High Throughput SHERLOCK Direct SARS-CoV-2 Test Workflow using NP swab eluted in Saline

.

A

SARS-CoV-2 Zeptometrix concentration in NP matrix(cp/µL)	Positive NO duplex	Positive RP
100	3/3	3/3
10	3/3	3/3
4	20/20	20/20
2	20/20	20/20
1	20/23	23/23
0.5	15/20	20/20
0.1	0/3	3/3
0	0/20	20/20

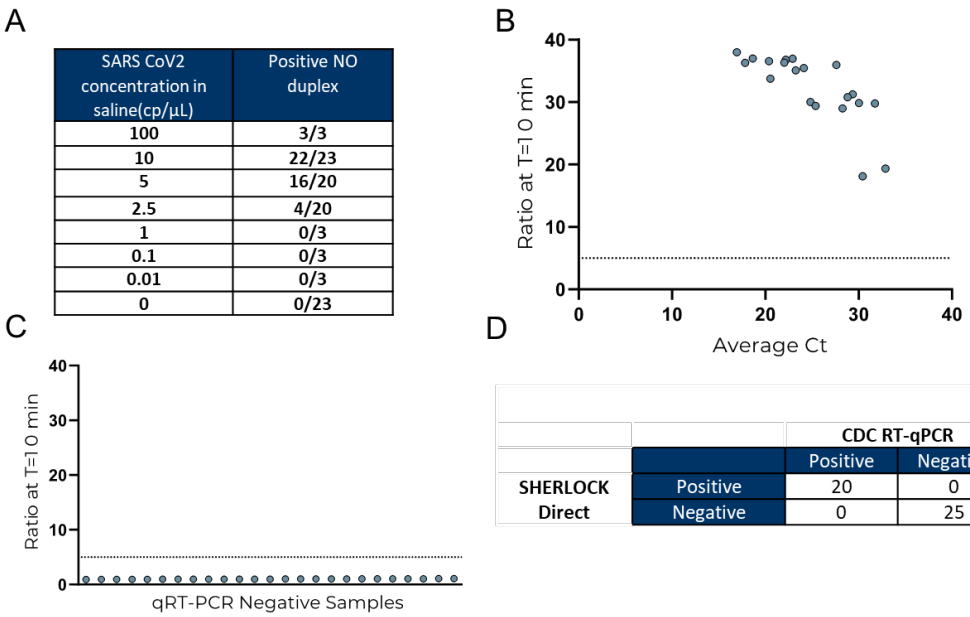


		CDC RT-qPCR	
		Positive	Negative
SHERLOCK HTP	Positive	30	0
	Negative	0	30

Figure 2. High-throughput SHERLOCK LoD and Clinical evaluation on Extracted NP samples. A) LoD using MagMAX Viral RNA isolation kit of negative NP matrix spiked with Zeptometrix NATtrol SARS-CoV-2 viral particles with 30min LAMP amplification step. B) LoD using negative nasal matrix spiked with heat-inactivated SARS-CoV-2 viral particles (FDA provided control material) with a 40 min LAMP amplification. C) 30 positive samples were tested by the CDC EUA method or SHERLOCK high-throughput method after MagMAX Viral RNA isolation. Each sample Ct value (average of N1 and N2 Ct values) was plotted against the ratio of the sample fluorescence to the NTC at 10 minutes after the addition of the Cas detection mix. Dotted line represents a ratio of 5, the cutoff for a positive sample. D) 30 negative clinical NP samples were extracted with the MagMAX Viral RNA Kit with a 30 min LAMP amplification step. Signal for each sample is reported as the fluorescence ratio of the sample divided by the florescence of the NTC 10 min after addition of Cas mixture to the LAMP reaction incubated at 37˚C Dotted line represents a ratio of 5, the cutoff for a positive sample.

A

SARS CoV2 concentration in saline(cp/µL)	Positive NO duplex
100	3/3
10	22/23
5	16/20
2.5	4/20
1	0/3
0.1	0/3
0.01	0/3
0	0/23



		CDC RT-qPCR	
		Positive	Negative
SHERLOCK Direct	Positive	20	0
	Negative	0	25

Figure 3. SHERLOCK Direct method on NP saline samples. A) LoD confirmation using NATtrol SARS-CoV-2 viral particles spiked into saline. Zeptometrix NATtrol SARS-CoV-2 viral particles were spiked into saline (0.9% NaCl) at indicated concentrations. Each sample was treated with Proteinase K and heated at 65˚C for 6 min and 98˚C for 3 min. 4µL of each sample was used for the SARS-CoV2 SHERLOCK assay using LAMP primers and Cas guides for the N gene and ORF1ab. Signal for each sample is reported as the fluorescence ratio of the sample divided by the florescence of the NTC 10 min after addition of Cas mixture to the LAMP reaction incubated at 37˚C. B) Clinical Evaluation of SHERLOCK Direct with positive NP swabs in saline. 20 positive clinical samples were tested by the CDC extractionless method or SHERLOCK direct method. Each sample Ct value (average of N1 and N2 Ct values) was plotted against the ratio of the sample fluorescence to the NTC at 10 minutes after the addition of the Cas detection mix. Dotted line represents a ratio of 5, the cutoff for a positive sample. C) 25 negative samples were confirmed by qRT-PCR, and their florescence ratios were determined by SHERLOCK Direct. D) The clinical agreement between each assa

Materials:

Table 1: Components of LAMP Reaction mix

Component	Supplier	Supplier – Catalog #
WarmStart® LAMP Kit (DNA & RNA)	NEB	E1700L
10x NO Primer Mix	IDT	Custom
10x RP Primer Mix	IDT	Custom

Table 2: Control Materials

Component	Supplier	Supplier – Catalog#	Description
SARS-CoV-2 Positive Control (*spc*)	BEI Resources ATCC®	NR-52285 VR1986D™	Genomic RNA from SARS-Related Coronavirus 2, Isolate USA-WA1/2020 or equivalent (BEI NR-52285, or ATCC® VR1986D™), diluted to a concentration of 225 copies/µL
Negative Template Control (*ntc*)	Qiagen	1039480	Nuclease-free water for use in Any Molecular Biology application

Table 3: Direct Sample Materials

Component	Supplier	Supplier – Catalog#
Proteinase K Solution	NEB	P8107S

Table 4: Cas Reaction Master Mix Materials

Components for Cas Detection Mix	Supplier	Sub-component	Sub-component supplier	Supplier – Catalog #
Cas Detection Mix A	Prepared by user	LwaCas13a Enzyme, 500 ng/µL	IDT	Custom
		T7 Polymerase, 50 U/µL	NEB	M0251
		$MgCl_2$ 1M	Any	N/A

Cas Detection Mix B	Prepared by user	rNTP Mix, 25 mM of each rNTP	NEB	N0466
		RnaseAlert	IDT	11-04-02-03
		Murine Rnase Inhibitor, 40 U/µL	NEB	M0314
		NO – crRNA	IDT	Custom
		RP – crRNA	IDT	Custom

Table 5: Equipment

Equipment	Specification
Fluorescent Plate Reader	Capable of incubation at 37°C, Ex/Em 420/528 nm
PC running Microsoft Excel	Any

Table 6: Additional Equipment and Consumables

Equipment and Consumables	Manufacturer	Catalog No.
0.2 mL strip tubes	Any	
1.5 mL snap cap tubes, low bind and nuclease-free	Any	
Molecular grade water (nuclease-free)	Any	
Molecular grade Mineral Oil	Sigma	69794
Dedicated adjustable P-10, P-20, P-100, P-200, and P-1000 pipettes for sample preparation	Any	
Dedicated adjustable P-10 or P-20 for dispensing template RNA	Any	
Dedicated adjustable P-10, P-20, P-100, P-200, and P-1000 pipettes for preparing and dispensing master mix	Any	
Dedicated adjustable M-10 and M-100 multichannel pipettes for transferring CRISPR Cas detection reaction	Any	
Dedicated electronic pipettes for dispensing master mixes (OPTIONAL)	Any	
Aerosol barrier tips	Any	
384 Well Corning Deep Well Plate	Corning	3575
Thermal Adhesive Sealing Film	Fisher Scientific	08-408-240
Biosafety Cabinet Class II, for the extraction	Any	
PCR Workstations, for each portion of the assay set up	Any	
Heat block with a heated lid able to fit a 384 deep well plate and capable of maintaining 61°C, 65°C and 98°C or PCR instrument with a heated lid	Any	
Vortex	Any	
Microcentrifuge	Any	
Cold blocks or ice	Any	
Tube racks	Any	
8 strip tube opener*	Any	

*recommended to reduce contamination risk

Experimental Protocol:

1. Reagent Controls and Preparation

a. SARS-CoV-2 Positive Control (*spc*) Preparation:

i. Precautions: This reagent should be handled with caution to prevent possible contamination. Freeze-thaw cycles should be avoided. Keep cold when thawed.

ii. Dilute the *spc* with nuclease free water to achieve the working concentration of 225 gene copies/µL. Make single use aliquots and store at ≤ -70°C.

iii. Thaw a single aliquot of the positive control for each experiment and keep on cold block until adding to the RT-LAMP reaction. Discard any unused portion of the aliquot

2. Extraction Methods

a. Proteinase K and Heat Treatment (without stabilization solution)

i. Aliquot 18 uL of NP/saline sample into PCR plate or strip tubes

ii. Add 2ul of Proteinase K to each sample.

iii. Heat at 65°C for 6 min, 98°C for 3 min, cool to 4°C prior to use in LAMP reaction.

b. Purelink Viral Mini kit (Cat 12280050)

i. Sample extraction can be performed using the PureLink™ Viral RNA/DNA Mini Kit (Cat. 12280050) as per the manufacturer's instructions with a 200 µL samples input volume, 30 µL elution volume.

c. MagMAX Viral Mini kit (A42352)

i. Sample extraction can be performed using the MagMAX™ Viral/Pathogen Nucleic Acid Isolation Kit (A42352) as per the manufacturer's instructions with a 200 µL samples input volume, 50 µL elution volume.

3. Procedure

a. **RT-LAMP Master Mix Preparation:**

i. Label a new 1.5 mL microcentrifuge tube for each target ("NO" for SARS-CoV-2 or "RP" for extraction control) and prepare a RT-LAMP Master Mix using the recipe in the table 1 blow for extracted RNA samples or table 2 for Proteinase K + heat treated samples.

ii. Pulse vortex RT-LAMP Master Mix for 3 seconds and spin down for 3 seconds in a microcentrifuge after all components are added.

Table 1: Target Specific RT-LAMP Master Mix Recipe for extracted RNA

Reagent	Volume per reaction	Volume total
2x RT-LAMP Mix	10 µL	10 µL x (*N* + 1)
10x Primer (SARS-CoV-2 or RP)	2 µL	2 µL x (*N* + 1)
Total Volume	12 µL	12 µL x *(N* + 1)

Table 2: Target specific RT-LAMP Master Mix Recipe for treated samples

Reagent	Volume per reaction	Volume total
2x RT-LAMP Mix	10 µL	10 µL x (*N* + 1)
10x Primer (SARS-CoV-2 or RP)	2 µL	2 µL x (*N* + 1)
Water	4 µL	4µL x *(N + 1)*
Total Volume	16 µL	16 µL x *(N* + 1)

iii. N = number of extracted samples plus number of controls. Prepare enough for 1 extra (N + 1) sample to allow for overage during reaction set-up.

b. **RT-LAMP Amplification in 384 well plate:**

i. Add 12 µL(if using extracted RNA) or 16µL(if using treated sample) of the RT-LAMP Master Mix into one well for each sample and control to be amplified.

ii. If using extracted RNA add 8 µL of the appropriate sample or control to the appropriate wells with LAMP master mix.

iii. If using Proteinase K + heat treated samples add 4µL of sample to the appropriate wells with LAMP master mix.

iv. Add 20µL of mineral oil to each of the wells.

Note: Add the mineral oil on the side of the well.

v. Seal the plate with Fisherbrand Thermal Adhesive Sealing Film

vi. Incubate the plate on the plate heater set to 61°C for 40 minutes. Cool to 21°C.

Table 3: RT-LAMP Assay Components and reaction volume

Reagent	Volume per reaction
RT-LAMP Master Mix	12 µL / 16µL
Extracted RNA Sample or Controls / Direct Saline	8 µL / 4 µL
Total Volume	20 µL

4. **CRISPR Cas Master Mix Preparation using Pre-mixed Components**

a. Prepare Cas Master Mix 1 and 2 according to table 4 below. These individual mixes can be stored at -20°C for up to 1 year.

b. Label a new 1.5 mL tubes with the target name CNO or CRP. Prepare CRISPR Cas Reaction Mix by combining appropriate volumes of Cas Master Mix 1, 2 and guide crRNA according to table 3 below. Once Master Mix 1 and 2 are combined, the mix should be used within 10 minutes.

Table 4: Target CRISPR Cas Master Mix Recipe using Pre-Mixed Components

Component	Reagent	Volume for each Reaction	Volume of Master mix in final reaction
Cas Master Mix 1	Cas enzyme (0.5mg/ml)	1.27 µL	1.77 µL
	T7 RNA Polymerase (50 U/µL)	0.5 µL	
Cas Master Mix 2	rNTP mix (25 mM of each rNTP)	1.00 µL	3.73 µL
	Rnase Alert (2nM)	1.56 µL	
	MgCl2 (1M)	0.23 µL	
	Murine Rnase Inhibitor (40 U/ µL)	0.63 µL	
	Water	0.31 µL	
Guide crRNA	NO crRNA (0.5µM each) or RP crRNA (0.5µM)	4.5 µL	N/A

i. Prepare enough for 1 extra (N + 1) sample to allow for overage during reaction set-up.

ii. Pulse vortex for 3 seconds and spin down for 3 seconds in a microcentrifuge after all components are added.

iii. Aliquot Cas Master mix into labeled PCR strip tubes to facilitate addition to 384 well plate.

5. **CRISPR Cas Detection**

CAUTION - Perform work in a unidirectional workflow in separate locations, from areas without specimen/nucleic acid or amplicon to areas with amplified nucleic acid.

a. Preheat plate reader to 37ºC.

b. Remove the 384 well plate with the RT LAMP amplification reactions from the plate heater.

c. Carefully remove the thermal seal from the 384 well plate. Change gloves.

d. Using a multichannel pipette and fresh tips for each transfer, transfer 10 µl of the CRISPR Cas mix into the appropriate wells of the plate (based on plate layout - Cas mix added to each RT LAMP amplification).

CAUTION - Do not go to the second stop of the pipette to avoid the introduction of bubbles to the reaction wells.

e. Seal the 384 well plate with optical seal.

f. Using BioTek Neo2 for reading Cas detection

i. Open the plate reader software to create a read procedure with the following settings:

ii. Set setpoint temperature to 37°C.

iii. Select “Kinetic” run reading with a total read time of 10 minutes, and data collection intervals at 5 minutes.

iv. Select filter settings in read details to 485nm/528nm filter set with the gains setting set to “extended”.

v. Set the read height to “9 mm” and the lamp energy to “high”

vi. Highlight the appropriate rows and columns based on plate template or read full plate in the plate settings.

vii. If a warning about “Max V” calculations appears, press “OK” and continue.

viii. Press green arrow to start, (i.e. “Create experiment and read now”).

6. **Results Extraction and Data Analysis**

a. After the completion of the plate reader run, select the wells that contain the samples. Export the data from these wells to an excel sheet.

b. For the negative template control (“*ntc*”), SARS-CoV-2 Positive Controls (“*spc*”) calculate the ratios as in Table 9 below:

Table 5: Ratio calculations for data analysis

Sample Type	Reaction Type	Reaction Name	Ratio Calculation
Negative Template Control (*ntc*)	SARS-CoV-2 [N and O gene targets]	NO^{ntc}	$\frac{NO_{t=10}^{ntc}}{NO_{t=0}^{ntc}}$
	Human RNaseP gene target	RP^{ntc}	$\frac{RP_{t=10}^{ntc}}{RP_{t=0}^{ntc}}$
SARS-CoV-2 Positive Control (*spc*)	SARS-CoV-2 [N and O gene targets]	NO^{spc}	$\frac{NO_{t=10}^{spc}}{NO_{t=0}^{spc}}$
Patient Specimen	SARS-CoV-2 target [N and O gene targets]	*NO*	$\frac{NO_{t=10}}{NO_{t=10}^{ntc}}$
	Human RNaseP gene target	*RP*	$\frac{RP_{t=10}}{RP_{t=0}^{ntc}}$

NO	= SARS-CoV2 N and O gene target reaction fluorescence
RP	= RNaseP target reaction
NO_{ntc}	= SARS-CoV2 N and O target negative template control reaction fluorescence
RP_{ntc}	= RNaseP target negative template control reaction fluorescence
NO_{spc}	= SARS-CoV2 N and O target positive template control reaction fluorescence
RP_{spc}	= RNaseP target positive template control reaction fluorescence
t	= reaction time on fluorescence plate reader (minutes)

7. Interpretation of Results

a. Assay Controls

i. **Negative Template Control Reactions (*ntc*):** Negative template control (*ntc*) reactions are used to monitor reagent and/or environmental contamination. There are two negative control reactions, one for each primer/crRNA set – (i) SARS-CoV-2 (ii) human RNaseP gene. Negative template control reactions are created by substituting the volume of sample material in the RT-LAMP reaction with an equal volume of nuclease-free water.

ii. **Positive Control Reactions (*spc*):** Positive control (*spc*) reactions are used to monitor gross reagent failure, such as reagent degradation, or incorrect assay set-up. There is one positive control reaction, one for SARS-CoV-2. Positive control reactions are created by substituting the volume of sample material in the RT-LAMP reaction with an equal volume of extracted SARS-CoV-2 viral RNA at a stock concentration of 225 copies/µL.

Table 6: Ratio calculations for data analysis

Control Type	Reaction Type	Reaction Name	Ratio Calculation	Ratio Calculation Result Interpretation	
				VALID	INVALID
Negative	NO target negative control	NO^{ntc}	$\frac{NO_{t=10}^{ntc}}{NO_{t=0}^{ntc}}$	< 3	≥ 3
	Human RNaseP gene target negative control	RP^{ntc}	$\frac{RP_{t=10}^{ntc}}{RP_{t=0}^{ntc}}$	< 3	≥ 3
Positive	SARS-CoV-2 N gene target positive control	NO^{spc}	$\frac{NO_{t=10}}{NO_{t=10}^{ntc}}$	≥ 5	< 5

NO = SARS-CoV2 N and O gene target reaction fluorescence

RP = RNaseP target reaction

NO_{ntc} = SARS-CoV2 N and O target negative template control reaction fluorescence

RP_{ntc} = RNaseP target negative template control reaction fluorescence

NO_{spc} = SARS-CoV2 N and O target positive template control reaction fluorescence

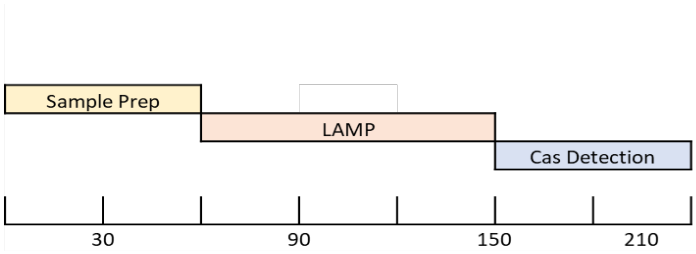
RP_{spc} = RNaseP target positive template control reaction fluorescence

t = reaction time on fluorescence plate reader (minutes)

Supplemental Figures

SHERLOCK High-throughput	
Step	Time
RNA extraction*	60 - 240 min
LAMP set up	15 min
LAMP	30 min
Cas set up	15 min
Cas rxn	10 min
Total 190 samples	**130 – 310 min**
SHERLOCK Direct High-throughput	
Add Prot K	45 min
Heat	10 min
LAMP set up	15 min
LAMP	40 min
Cas set up	15 min
Cas Rxn	10 min
Total 190 samples	**135 min**

Supplemental Table 1A. Comparison of processing time for SHERLOCK High-throughput and SHERLOCK Direct High-throughput. *60 minutes based on automated extraction.



Total minutes	210
Samples/cycle	4*190 = 760
Cycles per day	6
Samples/day	5211

Supplemental Table 1B. Estimated throughput of automated SHERLOCK High-throughput system

SARS-CoV-2 Zeptometrix concentration (cp/µL) in NP negative clinical samples	Zymo Quick DNA/RNA viral MagBead (R2140)		Mag MAX Viral/Pathogen (A42352)		MagMAX Viral RNA (AM1939)	
	NO	RP	NO	RP	NO	RP
100	3/3	3/3	3/3	3/3	3/3	3/3
10	3/3	3/3	3/3	3/3	3/3	3/3
1	1/3	3/3	3/3	3/3	2/3	3/3
0.1	0/3	3/3	0/3	3/3	0/3	3/3
0.01	0/3	3/3	0/3	3/3	0/3	3/3
0	0/3	3/3	0/3	2/3	0/3	3/3

Supplemental Table 2A. Comparison of 3 magnetic bead-based RNA extraction kits. The Zymo Quick DNA/RNA viral MagBead Kit, the MagMAX Viral/Pathogen kit and the MagMAX Viral RNA kit were compared for extraction of negative NP matrix spiked with Zeptometrix NATtrol SARS-CoV-2 viral particles with the SHERLOCK high-throughput workflow with 30min LAMP amplification step. Signal for each sample is reported as the fluorescence ratio of the sample divided by the florescence of the NTC 10 min after addition of Cas mixture to LAMP reaction. A sample was considered positive with a ratio of greater than or equal to 5.

SARS-CoV-2 genomic RNA concentration (cp/µL)	Bio-Tek Neo2	Tecan MPlex	Fluoroskan Microplate Fluorometer
180	3/3	3/3	3/3
90	3/3	3/3	3/3
45	3/3	3/3	3/3
22.5	3/3	3/3	3/3
11.25	3/3	3/3	3/3
10	3/3	3/3	3/3
8	3/3	3/3	3/3
4	3/3	3/3	3/3
2	3/3	0/3	3/3
1	3/6	4/6	1/6
0.5	1/3	0/3	0/3
0.1	0/3	0/3	0/3
0	0/6	0/6	0/6

Supplemental Table 2B. Comparison of three commercial plate readers.
The Biotek Neo2, Tecan MPlex and Fluoroskan Microplate Fluorometer were compared with the SHERLOCK high-throughput workflow for dilutions of SARS-CoV-2 genomic RNA. LAMP reactions were incubated for 30 min and NO duplex detected by the Cas detection reaction. Signal for each sample is reported as the fluorescence ratio of the sample divided by the florescence of the NTC 10 min after addition of Cas mixture to the LAMP reaction incubated at 37˚C. A sample was considered positive with a ratio of greater than or equal to 5.

Specificity			
Organism	**ATCC Cat. Number**	**Concentration**	**NO duplex**
Human coronavirus 229E	ATCC® VR-740D	6.67 x 10^5 copies/mL	0/3
Human coronavirus OC43	ATCC® VR-1558D	6.67 x 10^5 copies/mL	0/3
Human coronavirus HKU1	ATCC® VR-3262SD	6.67 x 10^5 copies/mL	0/3
Human coronavirus NL63	ATCC® 3263SD	6.67x 10^5 copies/mL	0/3
Influenza A	VR-95DQ	6.67 x 10^5 copies/mL	0/3
Influenza B	VR-1885DQ	6.67 x 10^5 copies/mL	0/3
Respiratory syncytial virus	ATCC® VR-1580DQ	6.67 x 10^5 copies/mL	0/3
Pseudomonas aeruginosa	ATCC® 27853D-5	6.67 x 10^6 copies/mL	0/3
Staphylococcus epidermis	ATCC® 12228D-5	6.67 x 10^6 copies/mL	0/3
Candida albicans	ATCC® 10231D-5	6.67 x 10^6 copies/mL	0/3
Human Reference RNA	Thermo Fisher QS0639	100 ng/rxn	3/3
Nuclease-free Water	Thermo Fisher R0581	N/A	0/3
SARS-CoV-2 genomic RNA	BEI NR-52285	1800-3600 cp/rxn	3/3

Supplemental Table 2C. Testing specificity of high-throughput SHERLOCK. Purified genomic DNA or RNA of potential cross-reactive high-risk pathogenic organisms of the respiratory tract were tested in triplicate by spiking into the NO duplex LAMP reaction followed by detection with Cas guides for the N and ORF1ab genes. All replicates were negative for SARS-CoV-2 detection.

Competitive Inhibition			
Organism	**ATCC Cat. Number**	**Concentration**	**NO duplex**
Human coronavirus 229E	ATCC® VR-740D	6.67 x 10^5 copies/mL	3/3
Human coronavirus OC43	ATCC® VR-1558D	6.67 x 10^5 copies/mL	3/3
Human coronavirus HKU1	ATCC® VR-3262SD	6.67 x 10^5 copies/mL	3/3
Human coronavirus NL63	ATCC® 3263SD	6.67x 10^5 copies/mL	3/3
Influenza A	VR-95DQ	6.67 x 10^5 copies/mL	3/3
Influenza B	VR-1885DQ	6.67 x 10^5 copies/mL	3/3
Respiratory syncytial virus	ATCC® VR-1580DQ	6.67 x 10^5 copies/mL	3/3
Pseudomonas aeruginosa	ATCC® 27853D-5	6.67 x 10^6 copies/mL	3/3
Staphylococcus epidermis	ATCC® 12228D-5	6.67 x 10^6 copies/mL	3/3
Candida albicans	ATCC® 10231D-5	6.67 x 10^6 copies/mL	3/3
Human Reference RNA	Thermo Fisher QS0639	100 ng/rxn	3/3
Nuclease-free Water	Thermo Fisher R0581	N/A	0/3
SARS-CoV-2 genomic RNA	BEI NR-52285	1800-3600 cp/rxn	3/3

Supplemental Table 2D. Testing for potential competitive inhibition of cross-reactive Organisms.
Purified genomic DNA or RNA from high-risk pathogenic organisms of the respiratory tract were tested in triplicate by spiking into the NO duplex LAMP reaction in the presence of SARS-CoV-2 genomic RNA. All replicates were positive for SARS-CoV-2 when detected with Cas guides for the N and Orf1ab genes.

Sample type	RT-qPCR		Sherlock	Sample type	RT-qPCR		Sherlock
	N1 Ct	N2 Ct	NO		N1 Ct	N2 Ct	NO
Negative	Undetermined	Undetermined	2.1	Positive	22.0	23.4	62.7
Negative	Undetermined	Undetermined	2.1	Positive	14.7	15.6	67.6
Negative	Undetermined	Undetermined	2.0	Positive	19.6	20.9	57.2
Negative	Undetermined	Undetermined	2.0	Positive	25.0	26.7	48.0
Negative	Undetermined	Undetermined	2.2	Positive	22.2	23.7	45.8
Negative	Undetermined	Undetermined	2.0	Positive	24.0	25.5	69.8
Negative	Undetermined	Undetermined	2.2	Positive	18.1	19.5	68.5
Negative	Undetermined	Undetermined	1.9	Positive	24.9	26.6	61.4
Negative	Undetermined	Undetermined	1.8	Positive	20.6	21.2	64.1
Negative	Undetermined	Undetermined	2.3	Positive	17.5	19.2	65.7
Negative	Undetermined	Undetermined	2.2	Positive	21.0	20.9	68.3
Negative	Undetermined	Undetermined	2.1	Positive	20.6	20.9	64.8
Negative	Undetermined	Undetermined	2.1	Positive	27.7	28.1	51.0
Negative	Undetermined	Undetermined	1.9	Positive	24.2	24.8	60.2
Negative	Undetermined	Undetermined	2.0	Positive	13.2	14.3	65.5
Negative	Undetermined	Undetermined	2.2	Positive	17.5	17.3	63.6
Negative	Undetermined	Undetermined	1.0	Positive	16.3	17.2	70.7
Negative	Undetermined	Undetermined	2.1	Positive	18.6	20.5	61.3
Negative	Undetermined	Undetermined	2.2	Positive	13.6	14.3	63.8
Negative	Undetermined	Undetermined	2.1	Positive	20.4	20.4	61.1
Negative	Undetermined	Undetermined	2.0	Positive	13.6	14.6	68.1
Negative	Undetermined	Undetermined	2.1	Positive	28.0	28.3	49.9
Negative	Undetermined	Undetermined	1.9	Positive	17.1	16.8	65.2
Negative	Undetermined	Undetermined	1.9	Positive	21.8	22.4	63.5
Negative	Undetermined	Undetermined	2.1	Positive	14.5	14.6	69.2
Negative	Undetermined	Undetermined	2.0	Positive	21.9	21.3	66.7
Negative	Undetermined	Undetermined	2.4	Positive	18.1	17.7	62.7
Negative	Undetermined	Undetermined	1.9	Positive	21.8	21.2	65.4
Negative	Undetermined	Undetermined	2.0	Positive	19.0	19.2	70.0
Negative	Undetermined	Undetermined	2.4	Positive	29.9	30.3	47.1

Supplemental Table 2E: 30 negative and 30 positive clinical samples were evaluated using RT-qPCR and SHERLOCK assays. CDC primers/probes for two regions of the SARS-CoV2 N gene (N1 and N2) were used to perform RT-qPCR and the Ct values are reported. For the SHERLOCK assay primers/guides for the N genes and ORF1ab (NO) were used and ratios of fluorescence signal of the samples compared to non-template control is reported.

Clinical Category	SHERLOCK Direct		CDC heat inactivation extractionless RT-qPCR			CDC QIAamp extraction RT-qPCR		
	NO duplex	RNaseP	N1 Ct	N2 Ct	RP Ct	N1 Ct	N2 Ct	RP Ct
positive	+	+	20.844	20.230	28.268	19.651	19.458	27.840
positive	+	+	18.033	17.616	28.097	17.508	17.076	29.222
positive	+	+	18.728	18.571	25.622	16.185	16.755	24.158
positive	+	+	30.344	29.723	24.791	28.118	28.874	25.251
positive	+	-	29.292	29.424	33.010	27.846	28.7	32.797
positive	+	+	17.062	16.840	28.653	15.555	15.653	28.369
positive	+	-	21.866	22.491	32.255	21.862	21.303	33.972
positive	+	-	21.377	22.692	27.894	18.412	20.469	28.051
positive	+	+	32.214	31.265	25.037	28.011	28.628	25.341
positive	+	+	20.214	20.546	28.847	19.642	18.993	30.756
positive	+	+	25.668	23.979	25.920	24.838	24.696	30.692
positive	+	+	23.261	22.595	24.374	22.457	22.301	26.727
positive	+	+	28.601	29.000	24.030	27.343	28.284	25.852
positive	+	+	30.247	30.631	23.215	29.467	30.423	25.305
positive	+	+	23.492	23.005	26.101	22.246	22.195	27.609
positive	+	+	24.800	25.989	24.411	22.696	24.639	25.892
positive	+	+	27.512	27.732	24.730	26.121	26.657	26.598
positive	+	+	32.460	33.295	24.387	32.137	33.094	26.395
positive	+	+	28.195	28.367	26.737	27.481	27.702	31.352
positive	+	+	23.950	24.276	25.602	18.970	21.274	27.045
negative	-	+	Undetermined	Undetermined	25.284	Undetermined	Undetermined	26.033
negative	-	+	Undetermined	Undetermined	25.959	Undetermined	Undetermined	25.968
negative	-	+	Undetermined	Undetermined	29.528	Undetermined	Undetermined	30.809
negative	-	+	Undetermined	Undetermined	29.963	Undetermined	Undetermined	30.900
negative	-	+	Undetermined	Undetermined	29.832	Undetermined	Undetermined	31.215
negative	-	+	Undetermined	Undetermined	28.644	Undetermined	Undetermined	29.844
negative	-	+	Undetermined	Undetermined	29.752	Undetermined	Undetermined	30.710
negative	-	+	Undetermined	Undetermined	31.152	Undetermined	Undetermined	31.399
negative	-	+	Undetermined	Undetermined	31.260	Undetermined	Undetermined	29.621
negative	-	+	Undetermined	Undetermined	30.350	Undetermined	Undetermined	29.200
negative	-	+	Undetermined	Undetermined	29.923	Undetermined	Undetermined	30.080
negative	-	+	Undetermined	Undetermined	31.546	Undetermined	Undetermined	30.090
negative	-	+	Undetermined	Undetermined	31.208	Undetermined	Undetermined	28.699
negative	-	+	Undetermined	Undetermined	31.497	Undetermined	Undetermined	30.858
negative	-	+	Undetermined	Undetermined	28.251	Undetermined	Undetermined	27.213
negative	-	+	Undetermined	Undetermined	31.149	Undetermined	Undetermined	28.964
negative	-	+	Undetermined	Undetermined	24.334	Undetermined	Undetermined	24.091
negative	-	+	Undetermined	Undetermined	30.724	Undetermined	Undetermined	29.025
negative	-	+	Undetermined	Undetermined	29.450	Undetermined	Undetermined	28.897
negative	-	+	Undetermined	Undetermined	32.312	Undetermined	Undetermined	29.338
negative	-	+	Undetermined	Undetermined	30.878	Undetermined	Undetermined	30.190
negative	-	+	Undetermined	Undetermined	30.779	Undetermined	Undetermined	29.676
negative	-	+	Undetermined	Undetermined	30.978	Undetermined	Undetermined	30.055
negative	-	+	Undetermined	Undetermined	30.028	Undetermined	Undetermined	29.708
negative	-	+	Undetermined	Undetermined	26.707	Undetermined	Undetermined	25.999

Supplemental Table 3A. 20 positive and 25 negative clinical NP swabs in 0.9% saline were tested for SARS-CoV-2 using three different methods. Each sample was tested by qRT-PCR using the CDC primers, following the CDC EUA protocol and Ct values of each sample is shown. The same samples were also treated with Proteinase K and heat then tested using SHERLOCK high-throughput SARS-CoV-2 assay.

SARS-CoV-2 Zeptometrix concentration in saliva (cp/µL)	Positive NO duplex	Positive MS2
20	32/32	31/32
10	35/35	35/35
5	32/32	32/32
2.5	31/32	32/32
1.25	3/8	8/8
1	0/3	3/3
0.1	0/3	3/3
0	0/35	35/35

Supplemental Table 3B. LoD of saliva tested with SHERLOCK Direct method. Zeptometrix NATtrol SARS-CoV-2 viral particles were spiked into pooled saliva at the listed concentrations. Each sample was treated with Proteinase K and heated at 65˚ for 6 min and 98˚ for 3 min. 10µL of each sample was used for the SARS-CoV-2 SHERLOCK assay using LAMP primers and Cas guides for the N and Orf1ab genes. MS2 was spiked into the heat and proteinase K treatment step and used as an internal control. Signal for each sample is reported as the fluorescence ratio of the sample divided by the florescence of the NTC 10 min after addition of Cas mixture to LAMP reaction. A sample was determined to be positive if the ratio was greater than or equal to 5.