Primerdesign™ Ltd

SNPsig® VariPLEX™ (Covid-19) Real-Time PCR assay

CE IVD

Instructions for Use (IFU)

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SNPSIG

SNPsig® VariPLEX™ (Covid-19)

In vitro Real-Time PCR diagnostic test for COVID-19 variants

Validated for Use with:

Sample Types	PCR Platform
Extracted RNA samples eluates from positive COVID-19 tests	Applied Biosystems® 7500 (Thermofisher) CFX Opus (Bio-Rad) and CFX Touch (Bio-Rad) – See section 12.2 for compatibility LightCycler® 480 II (Roche) genesig® q32 (Primerdesign, Novacyt Ltd)

96 tests

Advena Ltd,
Tower Business
Centre, 2nd Flr,
Tower Street, Swatar,
BKR 4013, Malta

D00060

Primerdesign™ Ltd, School Lane, Chandler's Ford, UK, SO53 4DG

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1 Intended Use

The SNPsig® VariPLEX™ (Covid-19) is a CE marked, *in vitro* diagnostic, real-time-reverse transcriptase PCR (Real-Time-PCR) multiplex assay intended for the allelic discrimination of severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) virus variants of concern 20I/501Y.V1, 20H/501Y.V2, 20J/501Y.V3 and 20C/S.452R, as well as biologically significant mutations N501Y and E484K. This product is part of a range of secondary reflex qualitative PCR tests to be conducted on the post-extraction sample eluates from which a positive COVID-19 test result has been achieved. Post-extraction eluates can be derived from saliva, nasal swabs, nasopharyngeal swabs and/or oropharyngeal swabs. This multiplex assay provides rapid screening of individuals suspected of SARS-CoV-2 variants of concern or as part of wider surveillance of biological significant mutations.

The assay has been designed to be used with Real-Time-PCR instruments capable of simultaneously detecting FAM (Max Absorption 499nm, Maximum Emission 519nm), HEX/VIC (Max Absorption 538nm, Maximum Emission 559nm), ROX (Max Absorption 575nm, Maximum Emission 602nm) and Cy5 (Max Absorption 643nm, Maximum Emission 667nm) fluorophores.

The SNPsig® VariPLEX™ (Covid-19) assay is intended for use by qualified and trained clinical laboratory personnel specifically instructed and trained in the techniques of Real-Time PCR and *in vitro* diagnostic procedures.

The assay has been validated for use with the designated PCR platforms listed in **Section 12.2**.

Positive results are indicative of the presence of SARS-CoV-2 variants 20I/501Y.V1, 20H/501Y.V2, 20J/501Y.V3 and 20C/S.452R, as well as biologically significant SNPs N501Y and E484K. Negative results do not preclude further mutational events for targets not assayed and should not be used as the sole basis for patient management decisions. Individual target confirmatory tests should be used to exclude co-infections. Positive and negative results must be combined with clinical observations, patient history, and epidemiological information.

Specimen test results are available to interpret in under three hours using the SNPsig® VariPLEX™ (Covid-19). This time includes the time to PCR setup, PCR run time, and availability of results.

2 Summary and Explanation

The novel coronavirus disease 2019 (COVID-19) pandemic, caused by SARS-CoV-2 virus, represents a major threat to health. COVID-19 has resulted in widespread morbidity and mortality. SARS-CoV-2 is known to have infected more than 100 million people (1). As the virus has spread across the world, mutations have arisen resulting in divergent clusters (clades) with different prevalence in different geographic regions (2,3).

Since the beginning of the pandemic, governments and scientists have applied sequencing technologies to identify mutations which alter the characteristics of the SARS-CoV-2, including mutations which alter transmissibility (4). These mutations occur within the spike protein and are predicted to alter the way that SARS-CoV-2 interacts with Angiotensin I converting enzyme 2 (ACE2) receptor, and other mutations on the spike protein associated with a reduced affinity for key neutralising antibodies (5-7).

On 18th December 2020, the UK announced the identification of the variant of concern (VOC) 202012/01, which is now also known as 20I/501Y.V1 and B.1.1.7 (8,9). This variant was identified in a large cluster of cases in Kent and North East London (8). This variant had an unusually large number of genetic changes, predominantly in the spike protein. One mutation of particular concern was N501Y, a key contact residue within the receptor-binding domain, which is associated with an increased binding affinity to human ACE2 (10-13). Since the announcement of this strain, two further VOCs carrying N501Y mutation have been identified. As of the 25th March 2021, 20I/501Y.V1 (B.1.1.7) has been seen in 125 countries, and 20H/501Y.V2 (B.1.351), originally identified in South Africa, and P.1 originally identified in Brazil, have been detected in 75 and 41 countries respectively (14-16). Recently, N501Y has been identified in a new variant under investigation (VUI) identified in California, USA, 20C/S.452R. These variants have each raised concern internationally around transmissibility, severity, antibody sneutralisation capabilities and potential impacts on vaccines (11).

In addition to N501Y, the VOCs 20H/501Y.V2 and P1 have been observed to share E484K, a mutation which raised particular concern because of its ability to evade antibodies (6,7,17). In the UK, recent independent analyses suggest that there might be a realistic possibility that infection with VOC 20I/501Y.V1 is associated with an increased risk of death compared to infection with non-VOC viruses (18) and this strain has not been implicated in vaccine efficacy (19,20). However, B.1.1.7 has recently been identified with the addition of E484K in a subset of cases, raising concerns regarding the likely significant impact of this mutation on vaccine efficacy (20-24). Another concerning mutation (L452R) has been identified in the VOC 20C/S.452R with evidence to support increased transmissibility and immune evasion with wide clinical implications (25-27). Targeted identification of these variants using unique identifiers will permit the capture of those concerning cases, identifying a majority of outbreaks associated with them.

With the increasing importance of identifying new VOC due to clinical implications related to treatment and vaccine resistance, new solutions for VOC detection are needed. Mutation surveillance using sequencing technologies have been central to the identification of VOC, however, delays in sequencing and interpretation of these results make it challenging to utilise sequencing in a rapid diagnostic setting where a VOC is believed to be responsible for an outbreak (28). Real-Time PCR based approaches can be used to rapidly identify VOCs and biologically significant mutations with additional advantage of being faster, cheaper and more easily implemented in a broad range of clinical settings.

3 Principles of the Procedure

For optimum product performance and sensitivity, SNPsig® VariPLEX™ (Covid-19) (CE IVD) should be used with an appropriate RNA extraction system.

Each genotyping primer/ probes contain between two and four labelled probes homologous to the genotypes under investigation. During PCR amplification of the reverse-transcribed target RNA, the probes will compete for binding across the variant region. The probe that is 100% homologous to the binding site will preferentially bind and give a fluorescent signal as PCR proceeds. It follows that the wild-type sequence will give a strong amplification plot through one channel whilst giving a very weak signal through the alternative channel. Variant samples will give an exactly inverse result. Most hardware platforms can perform this analysis automatically. The SNPsig® assays are compatible with all PCR instruments capable of detecting fluorescence in FAM, HEX/VIC, ROX and Cy5 emission channels, including selected genesig® family instruments.

SNPsig® VariPLEX™ (Covid-19) consists of 3 primer/ probes tubes with the format and channel allocations in table below.

Reagent Label	FAM	HEX/VIC	ROX	Cy5
VariPlex™ Tube 1 (Primer/ probes for E484K and 20I/501Y.V1)	E484K WT	E484K MUT	20I/501Y.V1 WT	20I/501Y.V1 MUT
VariPlex™ Tube 2 (Primer/ probes for 20H/501Y.V2 and 20J/501Y.V3) and Internal Control	WT	20H/501Y.V2 MUT	20J/501Y.V3 MUT	Internal Control (IC)
VariPlex™ Tube 3 (Primer/ probes for N501Y and 20C/S.452R)	N501Y WT	N501Y MUT	20C/S.452R WT	20C/S.452R MUT

*Wild Type (WT) = SARS-CoV-2 WT sequence for the target, Mutant (MUT) = mutant sequence specific of the variant of Concerns analysed

4 Materials Provided

The SNPsig® VariPLEX™ (Covid-19) assay contains:

Two silver foil packs of 3 primer/ probes tubes and mastermix each (48 reactions per pack)

Reagent Label	Number of Vials per pack	Lid Colour	Volume (µl per vial)	Resuspended with
VariPlex™ Tube 1 (Primer/ probes for E484K and 20I/501Y.V1)	1x 48 reactions	Amber cap	110 µl	Template Preparation Buffer
VariPlex™ Tube 2 (Primer/ probes for 20H/501Y.V2, 20J/501Y.V3 and Internal Control)	1x 48 reactions	Green Cap	110 µl	Template Preparation Buffer
VariPlex™ Tube 3 (Primer/ probes for N501Y and 20C/S.452R)	1x 48 reactions	Red Cap	110 µl	Template Preparation Buffer
Onestep Lyophilised Mastermix	3	Gold Cap	525 µl	Mastermix Resuspension Buffer

Positive controls in a red foil pack

Reagent Label	Number of Vials per pack	Lid Colour	Volume (µl per vial)	Resuspended with
VariPlex™ Positive Control Tube 1 (for E484K and 20I/501Y.V1)	1	Amber cap	500 µl	Template Preparation Buffer
VariPlex™ Positive Control Tube 2 (for 20H/501Y.V2 and 20J/501Y.V3)	1	Green Cap	500 µl	Template Preparation Buffer
VariPlex™ Positive Control Tube 3 (for N501Y and 20C/S.452R)	1	Red Cap	500 µl	Template Preparation Buffer

Reaction suspension buffers and water

Reagent Label	Number of Vials per pack	Lid Colour	Volume (µl per vial)	Resuspended with
Template Preparation Buffer	2	Yellow Cap	1500 µl	N/A
Mastermix Resuspension Buffer	6	Blue Cap	750 µl	N/A
RNase/DNase Free Water	1	White Cap	1500 µl	N/A

Internal control in a blue foil bag

Reagent Label	Number of Vials per pack	Lid Colour	Volume (µl per vial)	Resuspended with
Internal Control	1	Blue Cap	500 µl	Template Preparation Buffer

5 Required Equipment and Consumables (Not Provided)

- PCR hood
- Vortex
- Microcentrifuge (CL2 users)
- Adjustable micropipettes 2 or 10µl, 20µl, 200µl and 1000µl
- Aerosol barrier pipette tips with filters
- Disposable glove
- Scissors (optional)
- 10% Bleach (1:10 dilution of commercial 5.25-6.0% hypochlorite bleach)
- RNase/DNase remover
- PCR reaction plates (compatible with the RT-PCR instrument to be used)
- PCR plate seal (compatible with the PCR plate to be used)

6 Facilities/Training Requirements

Testing for the presence of SARS-CoV-2 RNA should be performed in an appropriately equipped laboratory by staff trained to the relevant technical and safety procedures:

- Refer to the UK Government guidance on handling and processing potential COVID-19 samples in laboratories: www.gov.uk/government/publications/wuhan-novel-coronavirus-guidance-for-clinical-diagnostic-laboratories/wuhan-novel-coronavirus-handling-and-processing-of-laboratory-specimens
- Refer to the World Health Organization Laboratory biosafety guidance related to coronavirus disease (COVID-19): Interim guidance, 28 January 2021: https://www.who.int/publications/i/item/WHO-WPE-GIH-2021.1
- Interim Laboratory Biosafety Guidelines for Handling and Processing Specimens Associated with SARS-CoV-2: https://www.cdc.gov/coronavirus/2019-nCoV/lab-biosafety-guidelines.html

7 Warnings and Precautions

7.1 General

- Handle all specimens as infectious material using safe laboratory procedures. Specimen processing should be performed in accordance with national biological safety regulations.
- Perform all manipulations of potential live virus samples within a class II (or higher) microbiological safety cabinet (refer to the guidance detailed in **Section 6**).
- Follow necessary precautions when handling specimens. Use personal protective equipment (PPE) consistent with current guidelines for the handling of potentially infectious samples.
- Use personal protective equipment such as (but not limited to) gloves, eye protection and lab coats when handling kit reagents while performing this assay and handling materials, including samples, reagents, pipettes and other equipment and reagents.
- Please consult the safety data sheet (SDS) before using this kit, which is available on request.

7.2 Preventing Contamination

- Amplification technologies such as PCR are sensitive to accidental introduction of PCR product from previous amplification reactions. Incorrect results could occur if either the clinical specimen or the real-time reagents used in the amplification step become contaminated by accidental introduction of amplification product (amplicon).
- The SNPsig® VariPLEX™ (Covid-19)-Variants Positive Controls are provided in a sealed foil envelope and contain a mixture of high copy number of synthetic DNA templates. It should be opened and processed away from test samples and kit components to avoid cross-contamination.
    - Maintain separate areas for handling of specimen preparation, pre-PCR assay setup, and post-PCR amplified nucleic acids.
    - Maintain separated, dedicated equipment (e.g. pipettes, microcentrifuge) and supplies (e.g. sample tubes, pipette tips) for handling of specimen preparation, pre-PCR assay setup, and post-PCR amplified nucleic acids.
    - Wear a clean lab coat and disposable gloves when setting up assays.
    - Change gloves regularly and whenever contamination is suspected.
    - Keep reagent and reaction tubes capped or covered as much as possible.
    - Change aerosol barrier pipette tips between all manual liquid transfers.
    - During preparation of samples, compliance with good laboratory techniques is essential to minimise the risk of cross-contamination between samples and

the inadvertent introduction of nucleases into samples during and after the extraction procedure. Good aseptic technique should always be used when working with nucleic acids.

- **DO NOT** substitute or mix reagent from different kit from other manufacturers. Use the appropriate buffers (provided with the kit) as instructed in the table in **Section 4**.
- Work surfaces, pipettes and centrifuges should be cleaned and decontaminated with cleaning products (e.g. 10% bleach and DNA/RNA remover) pre- and post-PCR set up to minimise risk of nucleic acid contamination.
- RNA samples should be maintained on a cold block or on ice during preparation to ensure sample stability.

- Handle post-amplification PCR plates/tubes with care to ensure that the seal is not broken.
- Dispose of unused kit reagents and human biological specimens according to national regulations (refer to guidance detailed in **Section 6**).

7.3 Prevent RNase/DNase contamination

- Use RNase/DNase free disposable plasticware and pipettes reserved for DNA/RNA work to prevent cross-contamination with RNases/DNases from shared equipment.
- Use RNase/DNase free filter tips throughout procedure to prevent aerosol and liquid contamination.

8 Reagent Storage, Handling and Stability Conditions

- The SNPsig® VariPLEX™ (Covid-19) assay is shipped at ambient temperatures but must be stored at -20°C upon arrival.
- The SNPsig® VariPLEX™ (Covid-19) assay should be stored in the original packaging and is stable for up to 6 months when stored at -20°C.
- Always check the expiration date prior to use. The kit should not be used past the "use by" date as indicated on the pack label and individual tube labels. Once the "use by" date has been reached, the kit components should be discarded following the disposal instructions in **Section 15**.
- If the kit's protective packaging is damaged upon receipt, please contact Primerdesign™ for instructions.
- All resuspended reagents are stable for one month when stored at -20°C.
- Repeated thawing and freezing should be kept to a minimum and should not exceed 5 freeze-thaw cycles. Once resuspended, components may be aliquoted into smaller volumes, if required.
- When in use the kit components should be returned to the freezer promptly after use to minimise the time at room temperature.
- Primer/ probes mixes, the enzyme mastermix, positive control template and RNA internal extraction control are all delivered lyophilised and must be resuspended in the appropriate supplied buffer to the correct volume as detailed in the table in **Section 4.**
- It is important to protect the fluorogenic primer/ probes mixes from light as this reagent is photosensitive.

9 Specimen Collection, Handling and Storage

9.1 Compatible Specimens

- SNPsig® VariPLEX™ (Covid-19) can be conducted on the extracted RNA sample eluates from positive COVID-19 tests.

9.2 Collecting the Specimen

- Inadequate or inappropriate specimen collection, storage and transport are likely to yield false test results. Training in specimen collection is highly recommended due to the importance of specimen quality. CLSI MM13 (Clinical and Laboratory Standards Institute) may be referenced as an appropriate resource. Alternatively, please refer to **Section 6** specimen collection guidance.

9.3 Transporting Specimens

- Specimens must be packaged, shipped and transported according to the current edition of the International Air Transport Association (IATA) Dangerous Goods Regulation. Follow shipping regulations for UN 3373 Biological Substance, Category B when sending potential SARS-CoV-2 specimens.

9.4 Storing Specimens

- Extracted nucleic acid should be stored at -70°C or lower.
- Refer to **Section 6** weblinks for guidance.

10 Reagent and Controls Preparation

10.1 OneStep Lyophilised Mastermix preparation

- Upon receipt, the dried mastermix can be stored at -20°C for up to 6 months or the expiry date, whichever occurs first.
- Using aseptic technique, resuspend in 525 µl of Mastermix Resuspension Buffer, gently swirl to mix.
- Resuspended mastermix is stable for up to one month when stored at -20° C.
- Freeze/ thaw cycles should be minimised and not exceed 5 freeze/thaws. The reagent once resuspended can be aliquoted into smaller volumes if required and stored at -20° C.

10.2 SNPsig® VariPlex™ (COVID-19) Primer/Probe mix preparation

- Upon receipt, the dried primer/ probes can be stored at -20° C for up to 6 months or the expiry date, whichever occurs first.
- Precautions: this reagent should only be handled in a clean area and not exposed to light.
- Using aseptic technique, resuspend the dried primer/ probes in 110µl (per each vial) of Template Preparation Buffer and vortex to mix.
- Resuspended primer/ probes are stable for up to one month when stored at -20°C.
- Freeze/ thaw cycles should be minimised and not exceed 5 freeze/thaws. The reagent once resuspended can be aliquoted into smaller volumes if required and stored at -20°C.
- Store aliquots in the dark and keep away from sunlight.

10.3 SNPsig® VariPLEX™ (Covid-19) Variants Positive control template preparation

- The SNPsig® VariPLEX™ (Covid-19) Positive control templates (PCT) are provided in a sealed foil envelope. Each of the PCT tube contains a mixture of high copy number synthetic DNA material and should be handled with caution in a dedicated nucleic acid handling area to prevent possible contamination of other kits reagents and clinical specimens.
- Upon receipt, the dried PCT can be stored at -20°C for up to 6 months or the expiry date, whichever occurs first. Do not use after the expiry date (see product label).
- Using aseptic technique, resuspend each of the dried PCTs in 500 µl of Template Preparation Buffer, vortex to mix. Resuspended PCT is stable for up to one month when stored at -20°C. Following resuspension, each this will be at a concentration of 2 x 10^5 copies per µl.

- Freeze/ thaw cycles should be minimised and not exceed 5 freeze/thaws. The reagent once resuspended can be aliquoted into smaller volumes if required and stored at -20°C.

10.4 SNPsig® VariPLEX™ (Covid-19) Variants RNA Internal control (IC) preparation

- The SNPsig® VariPLEX™ (Covid-19) RNA IC can be added to the nucleic acid extraction system (not provided) to provide an RNA template control, detect PCR inhibition and confirm the integrity of the PCR run.
- Precautions: This reagent should be handled with caution in a dedicated nucleic acid handling area to prevent possible contamination.
- Upon receipt, the dried IC can be stored at -20°C for up to 6 months or the expiry date, whichever occurs first.
- Using aseptic technique, resuspend the dried IC in 500 µl of Template preparation buffer, vortex to mix. Resuspended IC is stable for up to one month when stored at -20°C.
- Freeze/ thaw cycles should be minimised and not exceed 5 freeze/thaws. The reagent once resuspended can be aliquoted into smaller volumes if required and stored at -20°C.
- The IC is only in tube 2, with primer/ probes in the SNPsig® VariPLEX™ (Covid-19) Tube 2 (primer/ probes for 20H/501Y.V2, 20J/501Y.V3 and Internal Control).
- Successful Real-Time -PCR amplification of the IC indicates that PCR inhibitors are not present at a high concentration.
- Amplification of the control cDNA does not interfere with detection of the target gene(s) even when the target gene is present at low copy number. If a positive result is obtained, this indicates that the reaction was successful and that any negative result for the primary reaction is a true negative.
- As IC is only in tube 2 of assay, a negative result for the Cy5 channel in tube 2 is suggestive of a fault in the reaction and therefore negative results for other tubes (1 and 3) may be false negatives.

10.5 No Template Control (NTC) preparation

- RNase/DNase Free Water is a provided to use as a No Template control (NTC), if required, in addition to the NTC
- The NTC is used to check for contamination during PCR plate setup.
- For Tube 2, the NTC will contain an internal control which will be detected in the Cy5 channel (618-660).

11 General Preparation

- Clean and decontaminate all work surfaces, pipettes, centrifuges, and other equipment prior to use.
- Decontamination agents should be used such as 10% bleach, 70% ethanol, and an RNase/DNase remover to minimise the risk of nucleic acid contamination.
- Performance of the SNPsig® VariPLEX™ (COVID-19) assay is dependent upon the amount and quality of RNA extracted from specimens.

12 Assay Set Up

12.1 Mastermix Setup

Each of the three primer/ probes tubes in this assay must be treated separately for plate setup, i.e. each patient sample, PTC and NTC must be tested against each primer/ probes. Therefore, follow the below mastermix setup instructions for each of the three tubes.

a) Resuspend each of the primer/ probes tubes in 110 µl of Template Preparation Buffer, vortex to mix.

b) Resuspend the OneStep Lyophilised Mastermix in 525 µl Mastermix Resuspension Buffer, gently swirl to mix.

c) Plate setup configuration can vary with the number of specimens. NTC should be included in each genotyping reaction mix (refer to **Section 10.5** on how to prepare NTC).

d) A PCT must be included in each plate setup for each genotyping reaction mixture.

- The PCT will be added after all other reagents and samples have been added to the plate.
- This will be in an area for handling nucleic acid and away from the NTC and any clinical specimen/ samples.
- This is to prevent plate setup, reagent, or specimen contamination with the PCT.

e) Determine the number of reactions (n) to set up per assay (including NTC and PCT for each plate). It is necessary to make excess reaction mix to allow for pipetting error. Use the following guide to determine volume of reagents to add to the reaction mix:

- If number of samples (n) is ≤ 10, then N = n+1
- If number of samples (n) is > 10 and ≤ 20, then N = n+2
- If number of samples (n) is > 20, then N = n+ 10% of total number of samples

f) Prepare three reaction mixes, one for each primer/ probes. Label three 1.5ml RNase/DNase free tubes, i.e. Tube 1, 2 and 3. Dispense the following resuspended components into the correct labelled tube:

For Tube 1 and 3:

Reaction mix Component	1 x volume required (µl) *
Onestep Lyophilised Mastermix	10 µl*
Primer/ probes (either Tube 1 or 3)	2 µl*
RNase/DNase Free Water	3 µl*
Final Volume	**15 µl**

*Multiply all numbers by (N). Refer to step (e) above, to ensure there is sufficient reaction mix.

For Tube 2:

Reaction mix Component	1 x volume required (µl) *
Onestep Lyophilised Mastermix	10 µl*
Primer/ probes (Tube 2)	2 µl*
Internal Control Template	0.5 µl*
RNase/DNase Free Fater	2.5 µl*
Final Volume	**15 µl**

*Multiply all numbers by (N). Refer to step (e) above, to ensure there is sufficient reaction mix.

g) Add 15 µl of each genotyping reaction mix into the number of wells required for your testing, in an appropriate 96 well plate for your chosen PCR platform. Include 3 wells for the PCT, 3 wells for the NTC for each run.

h) Add 5 µl of the NTC into the appropriate wells according to your plate setup.

i) Cover the entire plate and move the plate to the specimen nucleic acid handling area.

j) Gently vortex nucleic acid sample tubes for approximately 5 seconds.

k) Centrifuge for 5 seconds to collect contents at the bottom of the tube, and then place the tube in a cold rack.

l) Change gloves often and when necessary, to avoid contamination.

m) Add 5 µl of the RNA/nucleic acid extracted from clinical specimen/sample(s) into the appropriate wells according to your plate setup.

n) Cover the entire plate and move the plate to the positive template control handling area.

o) Add 5 µl of PCT (please refer to **Section 10.3**) into the appropriate wells according to your plate set up. Seal the plate with an appropriate seal and place in the instrument.

12.2 Programming the Real-Time PCR Instrument

Please refer to one of the following manuals for additional information on using the instrument:

- Applied Biosystems® 7500 Real-Time PCR system Relative Standard Curve and Comparative Ct Experiments (as per Applied Biosystems® 7500 manual (2010)).
- LightCycler® 480 instrument Operator's manual (July 2016, Addendum 4, Software version 1.5)
- CFX Opus Real-Time PCR Instrument Guide (as per Bio-Rad Laboratories Inc. Manual (2017), software version CFX Maestro 2.0) and CFX Touch (as per comparability study[29]).
- genesig® q32 User Guide (2022) software version 1.5 or above.

a) Enter the following amplification program:

Steps	Time	Temperature	Cycles	Detection Format
Reverse Transcription	10 min	55°C	1	FAM (465-510)
Initial denaturation and Taq activation	2 min	95°C	1	HEX/VIC (533-580)
Denaturation	10 sec	95°C	45	ROX (533-610)
Annealing and extension*	60 sec	60°C*		Cy5 (618-660)

*Acquisition must be performed at the end of this stage.

13 Interpretation of Results

13.1 Acceptance criteria of controls included in the SNPsig® VariPLEX™ (Covid-19) assay

Before interpreting sample results, it is necessary to verify the success of the run. If the following criteria are not satisfied, then testing needs to be repeated:

- NTC is free from amplification in the FAM (499-519), HEX/VIC (538-559), ROX (575-602) and Cy5 (643-667) for Tube 1 and 3. NTC should produce a positive amplification in Cy5 (643-667) but be free from amplification in the FAM (499-519), HEX/VIC (538-559) and ROX (575-602) for tube 2***

- PCT produces a Cq of between 14-22 in the FAM (499-519), HEX/VIC (538-559), ROX (575-602 and Cy5 (643-667) channels.

***If the Negative Control does produce positive amplification in the FAM, Cy5 (except tube 2), HEX/VIC and ROX channel, the Cq value produced by the patient sample should be >5Cq earlier than the Negative Control (i.e. patient sample Cq =30, Negative Control Cq ≥35 is acceptable) in order to proceed with the interpretation of patient specimen results. However, if the patient sample produces a Cq <5Cq earlier than the Negative Control Cq (e.g. patient sample Cq =30, Negative Control Cq =32), then the results should not be analysed due to contamination.

Please manually inspect amplification curves for all samples assigned a Cq value to verify the positive amplification.

For instrument specific guidance on correctly assigning Cq values follow manufacture instructions.

13.2 Interpretation of Patient Specimen Results

If all the control acceptance criteria are fulfilled, then each sample can be assessed with the following metric.

Summary of mutations associated with known Variants of Concern (VOC) and Variants of Interest (VOI).

	Originally identified in	Mutation Specific Test					
		N501Y	UK Identifier	E484K	SA Identifier	Brazil Identifier	Cal Identifier
20I/501Y.V1	UK	X	X	-	-	-	-
20I/501Y.V1 with E484K	UK	X	X	X	-	-	-
20H/501Y.V2	South Africa	X	-	X	X	-	-
20J/501Y.V3	Brazil	X	-	X	-	X	-
20C/S.452R	California, USA	-	-	-	-	-	X

Tube 1: E484K and 20I/501Y.V1 reaction mix*:

E484K		20I/501Y.V		Result
WT	MUT	WT	MUT	
FAM	HEX/VIC	ROX	Cy5	
Cq (+)	Cq (-)	Cq (+)	Cq (-)	Original SARS-CoV-2
Cq (-)	Cq (+)	Cq (+)	Cq (-)	E484K Positive
Cq (+)	Cq (-)	Cq (-)	Cq (+)	20I/501Y.V1 Positive
Cq (-)	Cq (+)	Cq (-)	Cq (+)	20I/501Y.V1 with E484K Positive

*For any additional fluorescence profiles, we suggest sending the sample for sequencing. Missing fluorescence may indicate acquisition of additional mutations.

Tube 2: 20H/501Y.V2 and 20J/501Y.V3 reaction mix*:

20H/501Y.V2 and 20J/501Y.V3			Internal Extraction Control	Result
WT	20H/501Y.V2	20J/501Y.V3		
FAM	HEX/VIC	ROX	Cy5	
Cq (+)	Cq (-)	Cq (-)	Cq (+) / (-)	Original SARS-CoV-2
Cq (-)	Cq (+)	Cq (-)	Cq (+) / (-)	20H/501Y.V2 Positive
Cq (-)	Cq (-)	Cq (+)	Cq (+) / (-)	20J/501Y.V3 Positive

* For any additional fluorescence profiles, we suggest sending the sample for sequencing. Missing fluorescence may indicate acquisition of additional mutations.

Tube 3: N501Y and 20C/S.452R reaction mix*:

N501Y		20C/S.452R		Result
WT	MUT	WT	MUT	
FAM	HEX/VIC	ROX	Cy5	
Cq (+)	Cq (-)	Cq (+)	Cq (-)	Original SARS-CoV-2
Cq (-)	Cq (+)	Cq (+)	Cq (-)	N501Y Positive
Cq (+)	Cq (-)	Cq (-)	Cq (+)	20C/S.452R Positive

*For any additional fluorescence profile, we suggest sending the sample for sequencing. Missing fluorescence may indicate acquisition of additional mutations.

All instances of FAM (and ROX for tube 1 and 3) sample amplification for this assay indicates a SARS-CoV-2 positive sample. For the mutants test, all instances of HEX/VIC or Cy5 (or ROX for tube 2) channel sample amplification indicates a positive result for the respective mutation or variant. Please manually inspect amplification curves for all samples assigned a Cq value to verify the positive amplification.

14 Performance Evaluation

The SNPsig® VariPLEX™ (Covid-19) assay performance evaluation has been generated on the CFX Opus Real-Time PCR Instrument (Bio-Rad). A set of additional testing at the LoD level has been performed for the additional PCR instruments listed in **Section 12.2**.

14.1 Analytical Sensitivity

The limit of detection (LoD) is defined as the lowest concentration of analyte that could be reliably detected with >95% confidence. The LoD of the SNPsig® VariPLEX™ (Covid-19) was validated for each assay (E484K and 20I/501Y.V1, 20H/501Y.V2 and 20J/501Y.V3, N501Y and 20C/S.452R) across four PCR platforms (CFX Opus Real-Time PCR Instrument (Bio-Rad), Applied Biosystems® 7500 Real-Time PCR Instrument (Thermofisher), Lightcycler® 480 II Instrument (Roche) and genesig® q32 Real-Time PCR Instrument (Primerdesign).

Tentative LoD was established by using extracted negative saliva samples spiked with T7 run offs (RNA synthesised in vitro by T7 RNA polymerase during run-off transcription) for each assay (E484K and 20I/501Y.V1, 20H/501Y.V2 and 20J/501Y.V3, N501Y and 20C/S.452R) during the PCR setup. Saliva samples were extracted using the KingFisher Flex Purification System in conjunction with the exsig™ Mag extraction kit. The tentative LoD was tested at 3 contrivance levels: 0.5 (C1), 1 (C2) and 2 (C3) copies/µl for each assay which corresponds to 10, 20 and 40 copies/reaction, respectively. Each contrivance level was tested on 5 replicates. The LoD of an assay was considered as the LoD between WT and SNP template.

14.1.1 Verification of the LoD

Once the tentative LoD was established (95% positive call rate), it was verified by preparing the samples and contrivance (T7) in the same way as the tentative assay. T7 was diluted to required levels around the tentative LoD at each assay, giving a total of 20 data per target.

Assay 1			E484K (HEX/VIC)		WT (FAM)	
Mean Conc (copies/µl)	Mean Conc (copies/rxn)	Total replicates	Detection rate (%)	Mean Cq (STDV)	Detection rate (%)	Mean Cq (STDV)
0.5	10	20	20 (100)	32.20 (0.65)	20 (100)	33.65 (0.39)
0.25	5	20	18 (90)	34.73 (1.51)	19 (95)	35.11 (1.99)
0.125	2.5	20	19 (95)	34.86 (0.70)	20 (100)	35.51 (0.56)

For E484K, 19/20 replicates in the FAM channel amplified and 20/20 replicates in the HEX/VIC channel amplified for 2.5 copies/reaction. The LoD of this assay is therefore 2.5 copies/reaction.

Assay 2			20I/501Y.V1 (Cy5)		WT (ROX)	
Mean Conc (copies/µl)	Mean Conc (copies/rxn)	Total replicates	Detection rate (%)	Mean Cq (STDV)	Detection rate (%)	Mean Cq (STDV)
0.5	10	20	20 (100)	32.78 (0.28)	20 (100)	32.28 (0.41)
0.25	5	20	20 (100)	33.79 (0.26)	20 (100)	33.31 (0.53)
0.125	2.5	20	20 (100)	35.12 (0.37)	20 (100)	34.33 (0.73)

For 20I/501Y.V1, 20/20 replicates amplified in the FAM and HEX/VIC channel for 2.5 copies/reaction. The LoD is also 2.5 copies/reaction for this assay.

Assay 3			20J/501Y.V3 (ROX)		WT (FAM)	
Mean Conc (copies/µl)	Mean Conc (copies/rxn)	Total replicates	Detection rate (%)	Mean Cq (STDV)	Detection rate (%)	Mean Cq (STDV)
0.5	10	20	20 (100)	35.95 (0.56)	20 (100)	36.26 (0.55)
0.25	5	20	20 (100)	36.72 (0.77)	20 (100)	37.74 (0.89)
0.125	2.5	20	18 (90)	37.69 (1.01)	20 (100)	38.36 (1.07)

For 20J/501Y.V3, 18/20 replicates in the FAM channel for 2.5 copies/reaction, thus the LoD is higher. 20/20 replicates amplified in the ROX and FAM channel for 5 copies/reaction. The LoD for 20J/501Y.V3 is therefore 5 copies/reaction.

Assay 4			20H/501Y.V2 (HEX/VIC)		WT (FAM)	
Mean Conc (copies/µl)	Mean Conc (copies/rxn)	Total replicates	Detection rate (%)	Mean Cq (STDV)	Detection rate (%)	Mean Cq (STDV)
0.5	10	20	20 (100)	33.84 (0.53)	20 (100)	33.56 (0.46)
0.25	5	20	20 (100)	34.89 (0.87)	20 (100)	34.60 (0.52)
0.125	2.5	20	20 (100)	35.68 (1.12)	19 (95)	35.42 (0.46)

For 20H/501Y.V2, 20/20 replicates in the FAM channel amplified and 19/20 replicates in the HEX/VIC channel amplified for 2.5 copies/reaction. The LoD of this assay is 2.5 copies/reaction.

Assay 5			N501Y (HEX/VIC)		WT (FAM)	
Mean Conc (copies/µl)	Mean Conc (copies/rxn)	Total replicates	Detection rate (%)	Mean Cq (STDV)	Detection rate (%)	Mean Cq (STDV)
0.5	10	20	20 (100)	33.29 (0.48)	20 (100)	34.37 (0.28)
0.25	5	20	20 (100)	34.20 (0.89)	20 (100)	35.33 (0.46)
0.125	2.5	20	18 (90)	35.16 (3.57)	19 (95)	37.29 (0.94)

For N501Y, 18/20 replicates amplified in the FAM channel for 2.5 copies/reaction, thus the LoD is higher. 20/20 replicates amplified in the FAM and HEX/VIC channel for 5 copies/reaction. The LoD for this assay is 5 copies/reaction.

Assay 6			20C/S.452R (Cy5)		WT (ROX)	
Mean Conc (copies/µl)	Mean Conc (copies/rxn)	Total replicates	Detection rate (%)	Mean Cq (STDV)	Detection rate (%)	Mean Cq (STDV)
0.5	10	20	20 (100)	33.81 (0.82)	20 (100)	34.36 (0.30)
0.25	5	20	20 (100)	35.08 (0.42)	20 (100)	35.26 (0.32)
0.125	2.5	20	20 (100)	36.58 (0.61)	19 (95)	36.81 (0.67)

For 20C/S.452R, 20/20 replicates amplified in the ROX channel and the Cy5 channel for 2.5 copies/reaction. The LoD for this assay is 2.5 copies/reaction.

14.1.2 Alternative Instrument Testing

The LoD was further confirmed by testing on other three PCR platforms: Applied Biosystems® 7500 Real-Time PCR Instrument (Thermofisher), Lightcycler® 480 II Instrument (Roche) and genesig® q32 Real-Time PCR Instrument (Primerdesign). The LoD for each platform was determined at the copies/µl in the contrivance level in which product was detected at a 95% call rate.

Assay	LoD on CFX	LoD on q32	LoD on ABI	LoD on LightCycler 480
E484K	2.5 copies/reaction	10 copies/reaction	5 copies/reaction	5 copies/reaction
20I/501Y.V1	2.5 copies/reaction	5 copies/reaction	5 copies/reaction	2.5 copies/reaction
20H/501Y.V2	2.5 copies/reaction	10 copies/reaction	10 copies/reaction	2.5 copies/reaction
20J/501Y.V3	5 copies/reaction	10 copies/reaction	10 copies/reaction	5 copies/reaction
N501Y	5 copies/reaction	5 copies/reaction	10 copies/reaction	5 copies/reaction
20C/S.452R	2.5 copies/reaction	5 copies/reaction	5 copies/reaction	2.5 copies/reaction

Summary of the LoD for each assay on the different PCR platforms.

Assay	LoD on CFX	LoD on Q32	LoD on ABI	LoD on LightCycler 480
Tube 1	2.5 copies/reaction	10 copies/reaction	5 copies/reaction	5 copies/reaction
Tube 2	5 copies/reaction	10 copies/reaction	10 copies/reaction	2.5 copies/reaction
Tube 3	5 copies/reaction	5 copies/reaction	10 copies/reaction	5 copies/reaction

Summary of the LoD for each tube of the SNPsig® Variplex™ (COVID-19) assay on the different PCR platforms.

14.2 Analytical Specificity

The objective of this study is to assess the Analytical Specificity, i.e. inclusivity and exclusivity for the SNPsig® VariPLEX™ (COVID-19) assay. Exclusivity (cross-reactivity) was assessed by two methods. The first was via comprehensive in-silico analysis, and the second was to 'wet' test inactivated viruses and bacteria from related organisms using the SNPsig® VariPLEX™ (COVID-19) assay. The in-silico analysis also evaluated assay inclusivity.

14.2.1 Latest in silico specificity analysis

To ensure that COVID-19 primer/ probes remain specific to detect SARS-CoV-2 genomes, Primerdesign's Bioinformaticians daily review daily the SARS-CoV-2 sequence submissions on the GISAID EpiCoV database. As of 18th March 2021, in silico analysis confirms the COVID-19 assay primer/ probes still show 99.9% of the 623,394 sequences analysed at the ORF1ab target, and 99.8% of the 623,602 sequences analysed at the S gene target. The sequences were published on the GISAID EpiCoV database as of 18th March 2021 and include the variants of concern.

14.2.2 Wet testing

The following panels were used for the in vitro testing:

- NATrol™ Pneumonia Panel (ZeptoMetrix)
- NATtrol™ Coronavirus-SARS Stock
- Respiratory Evaluation Panel (Qnostics, Scotland, UK)
- QCMD panel from the 2019 Coronavirus EQA programme (Qnostics)
- QCMD panel from the 2019 MERS Coronavirus EQA Programme (Qnostics)
- SARS-CoV-2 Q Control (Qnostics)

Overall, the data obtained from Analytical Specificity demonstrates that the the SNPsig® VariPLEX™ (COVID-19) assay exhibits no cross reactivity with any of the panel members chosen for this study. None of the Coronavirus strains were detected in the Qnostics and Zeptometrix panels, whereas extracted SARS-CoV-2 strain was detected across all tested VariPLEX™ tubes in appropriate channels. Overall, this data confirms that the VariPLEX™ assay maintains the expected inclusivity and exclusivity criteria outlined in this study's Design Inputs.

		VariPLEX™ Tube1 Cq				VariPLEX™ Tube2 Cq			
Sample number	**Panel member**	**FAM**	**HEX/ VIC**	**ROX**	**Cy5**	**FAM**	**HEX/ VIC**	**ROX**	**Cy5 (IEC)**
1	A.baumannii	N/A	N/A	N/A	N/A	N/A	N/A	N/A	28.68
2	E.cloacae	N/A	N/A	N/A	N/A	N/A	N/A	N/A	25.12
3	E. coli	N/A	N/A	N/A	N/A	N/A	N/A	N/A	26.03
4	H.influenzae	N/A	N/A	N/A	N/A	N/A	N/A	N/A	28.01
5	K.aerogenes	N/A	N/A	N/A	N/A	N/A	N/A	N/A	27.92
6	K.oxytoca	N/A	N/A	N/A	N/A	N/A	N/A	N/A	26.36
7	M.catarrhalis	N/A	N/A	N/A	N/A	N/A	N/A	N/A	27.84
8	P.aeruginosa	N/A	N/A	N/A	N/A	N/A	N/A	N/A	26.94
9	P.mirabilis	N/A	N/A	N/A	N/A	N/A	N/A	N/A	27.58
10	S.agalactiae	N/A	N/A	N/A	N/A	N/A	N/A	N/A	27.39
11	S.aureus	N/A	N/A	N/A	N/A	N/A	N/A	N/A	28.25

12	S.marcescens	N/A	N/A	N/A	N/A	N/A	N/A	N/A	23.15
13	S.pneumoniae	N/A	N/A	N/A	N/A	N/A	N/A	N/A	26.88
14	S.pyogenes	N/A	N/A	N/A	N/A	N/A	N/A	N/A	27.77
15	K.pneumoniae	N/A	N/A	N/A	N/A	N/A	N/A	N/A	27.77
16	K.pneumoniae	N/A	N/A	N/A	N/A	N/A	N/A	N/A	27.14
17	K.pneumoniae	N/A	N/A	N/A	N/A	N/A	N/A	N/A	27.01
18	Coronavirus-SARS	N/A	N/A	N/A	N/A	N/A	N/A	N/A	28.00
19	INF A H1N1	N/A	N/A	N/A	N/A	N/A	N/A	N/A	27.32
20	INF A H3N2	N/A	N/A	N/A	N/A	N/A	N/A	N/A	27.52
21	INF B Victoria	N/A	N/A	N/A	N/A	N/A	N/A	N/A	27.35
22	INF B Yamagata	N/A	N/A	N/A	N/A	N/A	N/A	N/A	27.30
23	RSV A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	27.84
24	RSV B	N/A	N/A	N/A	N/A	N/A	N/A	N/A	26.94
25	Coronavirus- NL63	N/A	N/A	N/A	N/A	N/A	N/A	N/A	27.49
26	Coronavirus- 229E	N/A	N/A	N/A	N/A	N/A	N/A	N/A	27.33
27	Coronavirus- HKU	N/A	N/A	N/A	N/A	N/A	N/A	N/A	28.23
28	Coronavirus- OC48	N/A	N/A	N/A	N/A	N/A	N/A	N/A	27.51
29	MERS Coronavirus	N/A	N/A	N/A	N/A	N/A	N/A	N/A	27.37
Positive extraction control	Extracted SARS-CoV-2 Medium Q Control (mean)	35.02	N/A	33.68	N/A	35.07	N/A	N/A	27.56
PCT	VariPLEx™ Positive control	18.52	19.08	19.33	18.54	18.44	19.08	18.57	N/A
NTC	NTC	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A

Cq values obtained for VariPLEX™ Tube 1 and Tube 2.

		VariPLEX™ Tube3 Cq			
Sample number	**Panel member**	**FAM**	**HEX/VIC**	**ROX**	**Cy5**
1	A.baumannii	N/A	N/A	N/A	N/A
2	E.cloacae	N/A	N/A	N/A	N/A
3	E. coli	N/A	N/A	N/A	N/A
4	H.influenzae	N/A	N/A	N/A	N/A
5	K.aerogenes	N/A	N/A	N/A	N/A
6	K.oxytoca	N/A	N/A	N/A	N/A
7	M.catarrhalis	N/A	N/A	N/A	N/A
8	P.aeruginosa	N/A	N/A	N/A	N/A
9	P.mirabilis	N/A	N/A	N/A	N/A
10	S.agalactiae	N/A	N/A	N/A	N/A
11	S.aureus	N/A	N/A	N/A	N/A
12	S.marcescens	N/A	N/A	N/A	N/A
13	S.pneumoniae	N/A	N/A	N/A	N/A
14	S.pyogenes	N/A	N/A	N/A	N/A
15	K.pneumoniae	N/A	N/A	N/A	N/A
16	K.pneumoniae	N/A	N/A	N/A	N/A
17	K.pneumoniae	N/A	N/A	N/A	N/A
18	Coronavirus-SARS	N/A	N/A	N/A	N/A
19	INF A H1N1	N/A	N/A	N/A	N/A
20	INF A H3N2	N/A	N/A	N/A	N/A
21	INF B Victoria	N/A	N/A	N/A	N/A
22	INF B Yamagata	N/A	N/A	N/A	N/A
23	RSV A	N/A	N/A	N/A	N/A
24	RSV B	N/A	N/A	N/A	N/A
25	Coronavirus- NL63	N/A	N/A	N/A	N/A
26	Coronavirus- 229E	N/A	N/A	N/A	N/A

27	Coronavirus- HKU	N/A	N/A	N/A	N/A
28	Coronavirus- OC48	N/A	N/A	N/A	N/A
29	MERS Coronavirus	N/A	N/A	N/A	N/A
Positive extraction control	Extracted SARS-CoV-2 Medium Q Control (mean)	32.89	N/A	33.55	N/A
PCT	VariPLEx™ Positive control	18.38	18.96	18.63	18.06
NTC	NTC	N/A	N/A	N/A	N/A

Cq values obtained for VariPLEX™ Tube 3.

14.3 Accuracy

Diagnostic accuracy of the SNPsig® VariPLEX™ (COVID-19) assay was determined by generating a Positive Percentage Agreement (PPA), Negative Percentage Agreement (NPA) and Overall Percentage Agreement (OPA) for the wild-type (WT) and mutant (SNP) targets of the VariPLEX™ assay.

This study was designed to blind test 30 WT samples and 30 SNP samples. A total of 90 samples were tested for each tube of VariPLEX™ primer/ probes mix. Briefly, saliva samples, negative for SARS-CoV-2 were collected from multiple donors and extracted with the Kingfisher™ Flex Purification System in conjunction with exsig™ Mag Extraction System. Samples were contrived with verification material (RNA synthesised in vitro by T7 RNA polymerase during run-off transcription) for the relevant mutant targets at 5x LOD reported in Analytical Sensitivity Report. For the detection of wild type strain, Twist synthetic SARS-CoV-2 whole genome RNA control was used.

The total positive and negative results were used to generate Overall Percentage Agreement (OPA), Positive Percentage Agreement (PPA) and Negative Percentage Agreement (NPA). See tables below.

SNPsig® VariPLEX™ (Covid-19) Tube 1:

E484K WT (FAM)		Randomised contrived samples		
		Positive	Negative	Total
SNPsig® VariPLEX™ (Covid-19)	Positive	29	0	30
	Negative	1	60	60
	Total	30	60	90

Agreement	Level
OPA	99%
PPA	97%
NPA	100%

E484K mutant (HEX/VIC)		Randomised contrived samples		
		Positive	Negative	Total
SNPsig® VariPLEX™ (Covid-19)	Positive	29	0	29
	Negative	1	60	61
	Total	30	60	90

Agreement	Level
OPA	99%
PPA	97%
NPA	100%

20I/501Y.V1 WT (ROX)		Randomised contrived samples		
		Positive	Negative	Total
SNPsig® VariPLEX™ (Covid-19)	Positive	30	0	30
	Negative	0	60	60
	Total	30	60	90

Agreement	Level
OPA	100%
PPA	100%
NPA	100%

20I/501Y.V1 mutant (Cy5)		Randomised contrived samples		
		Positive	Negative	Total
SNPsig® VariPLEX™ (Covid-19)	Positive	30	0	30
	Negative	0	60	60
	Total	30	60	90

Agreement	Level
OPA	100%
PPA	100%
NPA	100%

SNPsig® VariPLEX™ (Covid-19) Tube 2:

20H/501Y.V2 and 20J/501Y.V3 WT (FAM)		Randomised contrived samples		
		Positive	Negative	Total
SNPsig® VariPLEX™ (Covid-19)	Positive	30	0	30
	Negative	0	60	60
	Total	30	60	90

Agreement	Level
OPA	100%
PPA	100%
NPA	100%

20H/501Y.V2 mutant (HEX/VIC)		Randomised contrived samples		
		Positive	Negative	Total
SNPsig® VariPLEX™ (Covid-19)	Positive	30	0	30
	Negative	0	60	60
	Total	30	60	90

Agreement	Level
OPA	100%
PPA	100%
NPA	100%

20J/501Y.V3 mutant (ROX)		Randomised contrived samples		
		Positive	Negative	Total
SNPsig® VariPLEX™ (Covid-19)	Positive	30	0	30
	Negative	0	60	60
	Total	30	60	90

Agreement	Level
OPA	100%
PPA	100%
NPA	100%

SNPsig® VariPLEX™ (Covid-19) Tube 3:

N501Y wild type (FAM)		Randomised contrived samples		
		Positive	Negative	Total
SNPsig® VariPLEX™ (Covid-19)	Positive	30	0	30
	Negative	0	60	60
	Total	30	60	90

Agreement	Level
OPA	100%
PPA	100%
NPA	100%

N501Y mutant (HEX/VIC)		Randomised contrived samples		
		Positive	Negative	Total
SNPsig® VariPLEX™ (Covid-19)	Positive	30	0	30
	Negative	0	60	60
	Total	30	60	90

Agreement	Level
OPA	100%
PPA	100%
NPA	100%

20C/S.452R wild type (ROX)		Randomised contrived samples		
		Positive	Negative	Total
SNPsig® VariPLEX™ (Covid-19)	Positive	30	1	31
	Negative	0	59	59
	Total	30	60	90

Agreement	Level
OPA	99%
PPA	100%
NPA	98%

20C/S.452R mutant (Cy5)		Randomised contrived samples		
		Positive	Negative	Total
SNPsig® VariPLEX™ (Covid-19)	Positive	30	0	30
	Negative	0	60	60
	Total	30	60	90

Agreement	Level
OPA	100%
PPA	100%
NPA	100%

14.4 Precision

Assessment of repeatability (intra-PCR run) and reproducibility (inter-PCR run) of the SNPsig® VariPLEX™ (COVID-19) assay has been assessed. T7 run offs were used as templates for the SNPsig® variants. These were diluted down to three contrivance levels (reproducing high, medium and low viral load samples):

- A high viral load sample (15-fold LoD)
- A medium viral load sample (10-fold LoD)
- A low viral load sample (5-fold LoD)

They were then added to the reaction mix and tested with saliva eluates after Kingfisher extractions as the samples. 10 independent-sample replicates were obtained for each contrivance level and per primer/probe tube: Tube 1A, Tube 1B, Tube 2, Tube 3A and Tube 3B were tested. Two different operators performed the study over 2 days on two CFX Opus Real-Time PCR instruments. The precision was measured by reporting the Coefficient of Variance which were well below and accepted industrial standard of 9% for all studies.

14.4.1 Repeatability

Intra-PCR run precision (repeatability) was measured by analysing within-run variation of replicates. A total of 10 samples of each contrived level were run on a single plate on the CFX Opus Real-Time PCR instruments. The statistical analysis of imprecision (SD and % CE) is shown below:

Tube	Contrivance level	Channel	% Positive Calls	Mean Cq	Stdev	Coefficient of Variance (%)
Tube 1A	CL 1 (15 x LoD)	ROX	100	30.1	0.2	0.7
		Cy5	100	29.4	1.1	3.8
	CL2 (10 x LoD)	ROX	100	30.6	0.2	0.7
		Cy5	100	30.1	0.3	0.9
	Cl 3 (5 x LoD)	ROX	100	31.7	0.2	0.6
		Cy5	100	31.2	0.3	1.1
Tube 1B	CL 1 (15 x LoD)	FAM	100	29.9	0.3	0.9
		HEX/VIC	100	30.8	0.1	0.3
	CL2 (10 x LoD)	FAM	100	30.6	0.4	1.4
		HEX/VIC	100	31.0	0.2	0.8
	Cl 3 (5 x LoD)	FAM	100	31.5	0.4	1.1
		HEX/VIC	100	31.8	0.1	0.4
Tube 2	CL 1 (15 x LoD)	FAM	100	30.2	0.2	0.5
		HEX/VIC	100	30.9	0.4	1.3
		ROX	100	30.7	0.1	0.4
		Cy5	100	22.7	0.1	0.2
	CL2 (10 x LoD)	FAM	100	31.0	0.2	0.5
		HEX/VIC	100	31.4	0.6	1.8
		ROX	100	31.2	0.1	0.5
		Cy5	100	22.7	0.1	0.3

	Cl 3 (5 x LoD)	FAM	100	32.2	0.2	0.7
		HEX/VIC	100	32.4	0.7	2.0
		ROX	100	32.5	0.3	0.8
		Cy5	100	22.8	0.1	0.4
Tube 3A	CL 1 (15 x LoD)	FAM	100	30.6	0.2	0.7
		HEX/VIC	100	31.1	0.2	0.7
	CL2 (10 x LoD)	FAM	100	31.1	0.2	0.6
		HEX/VIC	100	31.6	0.2	0.5
	Cl 3 (5 x LoD)	FAM	100	32.1	0.3	0.8
		HEX/VIC	100	32.5	0.2	0.7
Tube 3B	CL 1 (15 x LoD)	ROX	100	30.9	0.1	0.4
		Cy5	100	30.6	0.1	0.3
	CL2 (10 x LoD)	ROX	100	31.7	0.3	1.1
		Cy5	100	31.5	0.4	1.2
	Cl 3 (5 x LoD)	ROX	100	32.5	0.3	1.0
		Cy5	100	32.3	0.3	1.0

14.4.2 Reproducibility

Reproducibility was measured by analysing the inter-run variation of the SNPsig® VariPlex™ (Covid-19) assay subjected to a variety of different conditions: inter-instrument, inter-operator, and inter-day.

- Inter-instrument reproducibility was determined by running four plates on two CFX Opus Real-Time PCR instruments. A total of 10 samples of each contrived level were run on each plate. The statistical analysis of imprecision (SD and % CE) is shown below:

Contrivance Level	Tube	Channel	% Positive Calls	Mean Cq	Stdev	Coefficient of Variance (%)
CL 1 (15 x LoD)	1A	ROX	100	29.4	0.2	0.7
		Cy5	100	28.9	0.5	1.7
	1B	FAM	100	31.0	0.4	1.1
		HEX/VIC	100	30.8	0.2	0.7
	2	FAM	100	30.3	0.2	0.7
		HEX/VIC	100	30.2	0.1	0.5
		ROX	100	30.3	0.2	0.6
		Cy5	100	21.7	0.1	0.5
CL 2 (10 x LoD)	1A	ROX	100	30.3	0.2	0.7
		Cy5	100	29.9	0.3	1.2
	1B	FAM	100	31.3	0.4	1.4
		HEX/VIC	100	31.3	0.4	1.1
	2	FAM	100	30.9	0.1	0.3
		HEX/VIC	100	31.0	0.1	0.4

		ROX	100	30.9	0.2	0.5
		Cy5	100	22.0	0.3	1.6
CL 3 (5 x LoD)	1A	ROX	100	30.6	0.6	2.0
		Cy5	100	30.1	0.9	3.1
	1B	FAM	100	31.9	0.6	1.7
		HEX/VIC	100	31.8	0.2	0.7
	2	FAM	100	32.0	0.4	1.2
		HEX/VIC	100	32.0	0.2	0.7
		ROX	100	31.9	0.2	0.8
		Cy5	100	21.9	0.3	1.5
CL 1 (15 x LoD)	3A	FAM	100	31.0	0.3	0.9
		HEX/VIC	100	31.3	0.3	1.0
	3B	ROX	100	31.0	0.2	0.7
		Cy5	100	30.7	0.2	0.6
CL 2 (10 x LoD)	3A	FAM	100	31.3	0.4	1.2
		HEX/VIC	100	31.6	0.3	1.0
	3B	ROX	100	31.6	0.3	0.9
		Cy5	100	31.4	0.3	0.8
CL 3 (5 x LoD)	3A	FAM	90*	31.9	0.6	1.7
		HEX/VIC	100	32.1	0.3	0.9
	3B	ROX	100	32.6	0.5	1.4
		Cy5	100	32.4	0.4	1.2

* Denotes where value below 95% positive calls. This is due to a read error in the CFX Opus instrument which has caused two amplification curves to be pushed below zero. Visual inspection of the curve indicated evidence that these wells have amplified.

- Inter-operator precision was completed by two different operators each ran a plate for Tube 1 and 2, and a plate for Tube 3 with SNPsig® VariPLEX™ (COVID-19) assay. Both operators used the same CFX Opus Real-Time PCR instrument, and the runs were completed on the same day. A total of 10 samples of each contrived level were run on each plate. The statistical analysis of imprecision (SD and % CE) is shown below:

Contrivance level	Tube	Channel	% Positive Calls	Mean Cq	Stdev	Coefficient of Variance (%)
CL 1 (15 x LoD)	1A	ROX	100	29.72	0.33	1.11
		Cy5	100	29.23	0.92	3.14
	1B	FAM	100	29.86	0.74	2.49
		HEX/VIC	100	30.62	0.47	1.55
	2	FAM	100	31.13	0.97	3.1
		HEX/VIC	100	31.87	1.11	3.5
		ROX	100	31.45	0.81	2.58
		Cy5	100	22.62	0.24	1.07

CL 2 (10 x LoD)	1A	ROX	100	31.76	1.41	4.45
		Cy5	100	31.08	1.75	5.63
	1B	FAM	100	30.71	0.55	1.79
		HEX/VIC	100	30.71	0.38	1.22
	2	FAM	100	31.24	0.54	1.72
		HEX/VIC	100	31.98	1.2	3.76
		ROX	100	31.52	0.54	1.71
		Cy5	100	22.84	0.5	2.17
CL 3 (5 x LoD)	1A	ROX	100	32.01	0.62	1.93
		Cy5	100	31.3	1.07	3.41
	1B	FAM	100	31.53	0.45	1.44
		HEX/VIC	100	31.83	0.24	0.77
	2	FAM	100	31.25	1	3.2
		HEX/VIC	100	31.55	1.12	3.56
		ROX	100	31.58	1	3.17
		Cy5	100	22.59	0.47	2.06
CL 1 (15 x LoD)	3A	FAM	100	30.48	0.22	0.71
		HEX/VIC	100	30.53	0.21	0.7
	3B	ROX	100	30.78	0.17	0.55
		Cy5	100	30.54	0.16	0.51
CL 2 (10 x LoD)	3A	FAM	100	31.04	0.17	0.56
		HEX/VIC	100	31.61	0.14	0.44
	3B	ROX	100	31.53	0.29	0.93
		Cy5	100	31.29	0.33	1.06
CL 3 (5 x LoD)	3A	FAM	100	31.9	0.32	0.99
		HEX/VIC	100	32.47	0.25	0.78
	3B	ROX	100	32.43	0.26	0.8
		Cy5	100	32.26	0.27	0.84

- Inter-day precision was conducted using 2 plates for Tube 1 and 2 (plate 1 and 5), and Tube 3 (plate 2 and 6). The inter-day precision was run on 2 consecutive days using the SNPsig® VariPLEX™ (COVID-19) assay. The same CFX Opus Real-Time PCR instruments were used by each operator, and the runs were completed on the same day. A total of 10 samples of each contrived level were run on each plate. The statistical analysis of imprecision (SD and % CE) is shown below:

Contrivance level	Tube	Channel	% Positive Calls	Mean Cq	Stdev	Coefficient of Variance (%)
CL 1 (15 x LoD)	1A	ROX	100	29.79	0.38	1.26
		Cy5	100	29.13	0.9	3.09
	1B	FAM	100	30.4	0.58	1.89
		HEX/VIC	100	30.71	0.11	0.37
	2	FAM	100	30.18	0.14	0.47
		HEX/VIC	100	30.6	0.47	1.53
		ROX	100	30.46	0.28	0.91
		Cy5	100	22.16	0.55	2.46
CL 2 (10 x LoD)	1A	ROX	100	30.35	0.3	0.99
		Cy5	100	29.93	0.36	1.19
	1B	FAM	100	31.04	0.6	1.93
		HEX/VIC	100	31.04	0.3	0.98
	2	FAM	95	30.9	0.14	0.46
		HEX/VIC	95	31.2	0.43	1.38
		ROX	100	31.05	0.22	0.71
		Cy5	100	22.44	0.25	1.11
CL 3 (5 x LoD)	1A	ROX	100	30.86	0.89	2.87
		Cy5	100	30.33	1.06	3.5
	1B	FAM	100	31.61	0.39	1.23
		HEX/VIC	100	31.76	0.17	0.55
	2	FAM	100	32.08	0.38	1.18
		HEX/VIC	100	32.22	0.54	1.69
		ROX	100	32.16	0.43	1.34
		Cy5	100	22.49	0.39	1.73
CL 1 (15 x LoD)	3A	FAM	100	31.2	0.37	1.19
		HEX/VIC	100	32.03	0.66	2.06
	3B	ROX	100	31.28	0.28	0.9
		Cy5	100	31.52	0.74	2.35
CL 2 (10 x LoD)	3A	FAM	100	31.59	0.33	1.03
		HEX/VIC	100	32.41	0.68	2.11
	3B	ROX	100	32.04	0.44	1.38
		Cy5	100	32.29	0.83	2.59
CL 3 (5 x LoD)	3A	FAM	100	32.25	0.78	2.41
		HEX/VIC	100	33.08	1.14	3.44
	3B	ROX	100	32.99	0.37	1.13
		Cy5	100	33.21	0.71	2.14

Overall, the precision of the SNPsig® VariPLEX™ (Covid-19) assay showed a coefficient of variance below 9% indicating low variability in assay performance. All plates had NTCs free from amplification in all tested channels (FAM, HEX/VIC, ROX and Cy5). Therefore, overall precision of the SNPsig® VariPLEX™ (Covid-19) assay was acceptable.

15 Disposal

Dispose of unused kit reagents, human specimens and sealed post-amplification plates as laboratory clinical waste according to local, state and federal regulations. Refer to **Section 6** for guidance weblinks.

16 Technical Support

For Technical support, please contact our dedicated technical support team on:

Phone: +44 (0) 800 0156 494

Email: support@primerdesign.co.uk

17 References

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18 Explanation of Symbols

Symbol	Explanation
	In vitro diagnostics
	Manufacturer
	Catalogue number
	Sufficient for number of tests
	Use by Date
	Temperature limit
	Consult Electronic Instructions for Use
	Batch Code
	Keep away from sunlight (primer/probe mix)
	Positive Control

EC REP EU Authorized Representative

-  Single Use

PRIMER
DESIGN

Primerdesign™ Ltd
York House, School Lane, Chandlers Ford, SO53 4DG

Phone: +44 (0) 800 0156 494

Email: enquiries@Primerdesign.co.uk

Website: www.primerdesign.co.uk

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