Instruction for Use

SNPsig® SARS-CoV-2 EscapePLEX™ CE
96 Tests

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CE

1. Intended Purpose

The qualitative SNPsig®-SARS-CoV-2-EscapePLEX-CE is a new product designed as a molecular in vitro diagnostic test for the allelic discrimination of the SARS-CoV-2 variants. The process type will follow industrially established standard real-time PCR method. This includes the universally used TaqMan technology within two different fluorescent channels, It will be used to identify the new SARS-CoV-2 E484K, K417T, K417N and P681R mutations. It will represent a range of secondary reflex qPCR tests to be conducted on the post-extraction sample eluates from which a positive COVID-19 test result has already been achieved. The tests are intended to be used on patient samples from people of all ages at any point during active infection which may be asymptomatic or symptomatic and differentiating from other respiratory infections to detect SARS-COV-2 positive patients, to facilitate mass population screening and will satisfy authorities requesting multiple target analysis.

The product is designed for professional use to be incorporated into the established molecular laboratory workflow of sample collection, sample extraction and qPCR.

It is expected that the post-extraction eluate will be derived from saliva, nasopharyngeal swabs and/or oropharyngeal swabs.

2. Summary

The novel coronavirus disease 2019 (COVID-19) pandemic, caused by SARS-CoV-2 virus, represents a major threat to health. SARS-CoV-2 has resulted in widespread morbidity and mortality. COVID-19 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 modify the characteristics of the SARS-CoV-2, including mutations which alter transmissibility (4) or that are associated with a reduced affinity for key neutralising antibodies (5-7). These mutations mostly 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.

Recently, four clinically significant mutations have been identified as “high risk” due to the concerning increase in numbers of active cases carrying them.

E484K has been detected in a wide variety of Variants of Concern (VOC) and Variants Under Investigation (VUI), including Beta (B.1.351) Gamma (P.1), VOC-21FEB-02 (Alpha (B.1.1.7) with E484K), Zeta (P.2) and Theta (P.3) (8-11). Multiple studies on E484K have associated this change in amino acid with the ability to evade antibodies (12-14), increase disease severity and its potential impact on vaccines (15-18) with enormous implications for public health approaches in combatting COVID-19 cases.

The site K417 in the spike protein corresponds with an area in the protein that is considered a hotspot for antibody recognition (19). Therefore, changes in this position would have a potential role in antibody and vaccine escape. At present, two mutations on this site have been recognised and associated with reduced antibody protection. K417T was firstly identified in the VOC P.1 (20) and K417N was firstly identified as part of the VOC Beta (3.1.351) (19-20). In addition, K417N has been demonstrated to provide the virus with a moderate increase in transmissibility rates due to the very specific change induced by the K to N amino acid replacement (21).

P681R has been found in the recently identified VOC and VUI Kappa (B.1.617.1), Delta (B.1.617.2) and B.1.617.3 related variants (22). This mutation has been associated with a potential increase in transmissibility rate based on mechanistic studies on the capacity of the virus to identify and infect human cells (22).

Identifying variants that carry any of these four mutations in a timely manner may be important for making public health decisions. The product will be developed in a standard SNPsig format. It will enable the identification of these mutations (E484K, K417N/T and P681R) on previously identified SARS-CoV-2 positive samples.

3. Test principle

Genotyping by Real-Time PCR using hydrolysis probes

Each genotyping primers/probe mix contain four (tube 1) and three (tube 2) labelled probes homologous to the genotypes under investigation and the Internal Control. The SNPsig® assays are compatible with all Real-Time PCR instruments capable of detecting fluorescence in FAM, HEX/VIC, ROX and Cy5 emission channels, including selected genesig® family instruments.

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Reagent Label	FAM	HEX/VIC	ROX	Cy5
SNPsig® SARS-CoV-2 (EscapePLEX) Tube 1	E484K	K417N	K417T	P681R
SNPsig® SARS-CoV-2 (EscapePLEX) Tube 2	Orf1ab region	Internal Control	M region	N/A

Positive control Template

The kit contains two positive control templates tubes. Tube 1 contains the four synthetic templates homologous to the clinically significant mutations E484K, K417N, K417N and P681R. Tube 2 contains synthetic templates homologous to the Orf1ab and M gene target regions. These should be run with the appropriate primer/probe tube to obtain control signals for each target. Positive control templates are a potential contamination risk to subsequent tests so must be handled carefully.

Negative control

To confirm the absence of contamination a negative control reaction should be included for each primer/probe tube, every time the kit is used. In this instance, the RNase/DNAse free water should be used instead of template RNA. A negative result in FAM, HEX/VIC, ROX and Cy5 channels indicates that the reagents have not become contaminated while setting up the run. If a positive result is obtained, this indicates contamination, therefore the sample results will be invalid, and the run should be repeated.

Possible sources of contamination should first be explored and removed.

Master mix compatibility

Onestep Lyophilised Master Mix contains the enzyme, nucleotides, buffers, and salts at precisely the correct concentration for this application. The annealing temperatures of the primers and probes have been carefully calibrated and any change in the reaction buffer can significantly alter the performance of the assay. For this reason, Primerdesign can only guarantee accurate genotyping results when Onestep Lyophilised Master Mix is used.

4. Materials

1 x EscapePLEX (SARS-COV-2) Tube 1 Primer/probe mix

1 X EscapePLEX (SARS-COV-2) Tube 2 Primer/probe mix

4 x Onestep Lyophilised Master Mix

1 x EscapePLEX (SARS-COV-2) positive control template Tube 1

1 x EscapePLEX (SARS-COV-2) positive control template Tube 2

1 x Internal control RNA

2 x Template preparation buffer

4 x Master mix resuspension buffer

1 x Water RNase/DNase free

This product has been validated on the genesig® q32 Real-time PCR platform, Bio-Rad CFX Opus Real-Time PCR system, Applied Biosystems 7500 Fast Real-Time PCR system, and Roche® Lightcycler 480 II.

5. Storage

Storage conditions

- The SNPsig® Real-Time PCR SARS-COV-2 EscapePLEX assay is shipped at ambient temperatures but must be stored at -20°C upon arrival.
- The SNPsig® Real-Time PCR SARS-COV-2 EscapePLEX assay should be stored in the original packaging and is stable for up to 6 months once stored at -20°C.
- Repeated thawing and freezing should be kept to a minimum and should not exceed 5 freeze-thaw cycles.
- If the kit's protective packaging is damaged upon receipt or the tamper proof seal has been compromised, please contact Primerdesign for instructions. Attention should be paid to the "use by" date specified on the pack label and individual tube labels. On this date, the kit should be discarded following the disposal instructions in Section 11.
- Always check the expiration date prior to use. Do not use expired reagents.
- Primer/probe mixes, the enzyme master mix, 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.
- Once resuspended, components may be aliquoted into smaller volumes, if required, and are stable for up to one month if stored at -20°C.
- It is important to protect the fluorogenic primer/probe mixes from light as this reagent is photosensitive.

In Use Stability

- The SNPsig® Real-Time PCR SARS-COV-2 EscapePLEX assay should be stored in the original packaging and is stable for up to one month once resuspended and stored at -20°C.
- The kit should not be used past the "use by" date as indicated on the pack label and
- individual tube labels.
- When in use the kit components should be returned to the freezer promptly after use to minimize the time at room temperature.
- Repeated thawing and freezing should be kept to a minimum and should not exceed 5 freeze-thaw cycles. Components may be aliquoted into smaller volumes after resuspension, if required.

6. Warnings

1. Please consult the Safety Data Sheet (SDS) before using this kit which is available on request.
2. Please comply with laboratory codes of practice.
3. For in vitro diagnostic use (IVD) only.
4. Handle all specimens as if infectious using safe laboratory procedures. Specimen processing should be performed in accordance with national biological safety regulations.
5. Perform all manipulations of potential live virus samples within a class II (or higher) biological safety cabinet (refer to the guidance detailed in Section 7).
6. Follow necessary precautions when handling specimens. Use personal protective equipment (PPE) consistent with current guidelines for the handling of potentially infectious samples.
7. 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.
8. The SNPsig® Real-Time PCR SARS-COV-2 EscapePLEX assay component "Template preparation buffer" contains EDTA. This component should be handled according to the SDS. In the event of damage to protective packaging, contact Primerdesign for instructions.

7. Specimen Collection and Handling

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.
- Refer to the UK Government guidance on handling and processing potential COVID-19 samples in laboratories: https://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 Interim guidance on laboratory biosafety from 13 May 2020: Laboratory testing for 2019 novel coronavirus (2019-nCoV) in suspected human cases: https://www.who.int/emergencies/diseases/novel-coronavirus-2019/technical- guidance/laboratory-guidance
- Refer to Interim Guidelines for Collecting, Handling and Testing Clinical Specimens from Persons under Investigation (PUIs) for Coronavirus Disease 2019 (COVID-19) https://www.cdc.gov/coronavirus/2019-nCoV/guidelines-clinical-specimens.html
- Follow specimen collection devices manufacturer instructions for proper collection methods.
- Swab specimens should be collected using swabs with a synthetic tip, such as nylon or Dracon® and with an aluminium or plastic shaft. Calcium alginate swabs are unacceptable and cotton swabs with wooden shafts are not recommended. Place swabs immediately into sterile tubes containing 2-3 ml of viral transport medium.

Transporting Specimens

- Specimens must be package, 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.

Storing Specimens

- Extracted nucleic acid should be stored at -70ºC or lower.
- Refer to Section 5.1 weblinks for guidance.

8. Assay procedure

Resuspension Protocol

To minimize the risk of contamination with foreign RNA/DNA, we recommend that all pipetting be performed in a PCR clean environment. Ideally this would be a designated PCR lab or PCR cabinet. Filter tips are recommended for all pipetting steps. The positive control template is a significant contamination risk and should therefore be pipetted after negative control and sample wells.

1. **Pulse-spin each tube in a centrifuge before opening.**

This will ensure that the lyophilised primer/probe mix is in the base of the tube and is not lost upon opening the tube.

2. **Resuspend the primer/probe mix in the RNase/DNase free water supplied, according to the table below.**

To ensure complete resuspension, vortex the tube thoroughly.

Component - Resuspend in water	Volume
SARS-CoV-2 **(EscapePLEX)** genotyping primer/probe mix **tube 1** (BROWN)	110 µl
SARS-CoV-2 **(EscapePLEX)** genotyping primer/probe mix **tube 2** (BROWN)	110 µl

3. **Resuspend the Master Mix in Resuspension Buffer supplied, according to the table below.**

Component - Resuspend in Resuspension Buffer	Volume
Onestep Lyophilised Master Mix (GOLD)	525 µl

4. **Resuspend the Positive Control Templates tube in the Template Preparation Buffer supplied, according to the table below.**

   To ensure complete resuspension, vortex each tube thoroughly.

Component - Resuspend in Template Preparation Buffer	Volume
Positive Control Template tube 1 (RED) *	500 µl
Positive Control Template tube 2 (RED) *	500 µl

* This component contains high copy number template and is a VERY significant contamination risk. It must be opened and handled in a separate laboratory environment, away from the other components.

5. **Resuspend the Internal Control Templates tube in the Template Preparation Buffer supplied, according to the table below.**

Component - Resuspend in Template Preparation Buffer	Volume
Internal Control Template (BLUE)	500 µl

Real-Time PCR detection protocol

1. **Prepare 2 reaction mixes, one for each primers/probes mix.**

2. **Include sufficient reactions for all samples, positive and negative controls.**

Reaction mix for Tube 1:

Component	Volume
Onestep Lyophilised Master Mix	10 µl
SARS-CoV-2 **(EscapePLEX)** genotyping primer/probe mix Tube 1	1 µl
RNase/DNase free water (WHITE)	4 µl
Final Volume	**15 µl**

Reaction mix for Tube 2:

Component	Volume
Onestep Lyophilised Master Mix	10 µl
SARS-CoV-2 **(EscapePLEX)** genotyping primer/probe mix Tube 2	1 µl
Internal Control	1 µl
RNase/DNase free water (WHITE)	3 µl
Final Volume	**15 µl**

3. **Pipette 15 µl of the appropriate reaction mix into each well according to your Real-Time PCR experimental plate set up, to enable each sample to be tested with**

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each reaction mix.

4. **Prepare RNA templates for each of your samples.**

   As this is a reflex test, the RNA obtained from the confirmatory primary assay for SARS- CoV-2 will be used.

5. **Pipette 5 µl of RNA template into each well, according to your experimental plate set up.**

6. **For negative control wells use 5 µl of RNase/DNase free water.**

7. **Pipette 5 µl of each positive control RNA according to your experimental plate set-up. The final volume in each well is 20 µl.**

Real-Time PCR amplification protocol

The following protocol is recommended for optimum resolution between genotypes:

Protocol for Onestep Lyophilised Master Mix

	Step	Time	Temp
	Reverse transcription	10 min	55ºC
	Enzyme activation	2 min	95ºC
Cycling x45	Denaturation	10 s	95ºC
	Annealing and extension*	60 s	60ºC

* Fluorogenic data should be collected during this step through the **FAM, HEX/VIC, ROX** and **Cy5** channels for tube 1 and **FAM, HEX/VIC** and **ROX** for tube 2.

Interpretation of results

The SNPsig® SARS-CoV-2 (EscapePLEX) allows simultaneous discriminatory identification of the four clinically significant mutations, E484K, K417N, K417T and P681R in tube 1. Tube 2 allows the confirmation of a SARS-CoV-2 positive sample.

In tube 1:

- E484K should be producing a fluorescence signal on FAM channel.
- K417N should be producing a fluorescence signal on HEX/VIC channel.
- K417T should be producing a fluorescence signal on ROX channel.
- P681R should be producing a fluorescence signal on Cy5 channel (see table below).

FAM (E484K)	HEX/VIC (K417N)	ROX (K417T)	Cy5 (P681R)	Results
Cq (+)	Cq (-)	Cq (-)	Cq (-)	Positive for E484K
Cq (+)	Cq (+)	Cq (-)	Cq (-)	Positive for E484K + K417N
Cq (-)	Cq (+)	Cq (-)	Cq (-)	Positive for K417N
Cq (-)	Cq (-)	Cq (+)	Cq (-)	Positive for K417T
Cq (+)	Cq (-)	Cq (+)	Cq (-)	Positive for E484K + K417T
Cq (-)	Cq (-)	Cq (-)	Cq (+)	Positive for P681R
Cq(-)	Cq (+)	Cq(-)	Cq (+)	Positive for P681R + K417N

Lack of fluorescence in a channel could either indicate a sample with Wild-type sequence at that site or a sample with a different mutation at the target site. Combination of mutation (Cq (+) in more than one channel) is possible. For any additional fluorescence profiles, we suggest sending the sample for sequencing.

Due to the evolution of new variants and the extinction of others, the variants associated with the mutation profiles in the above table have not been added. For the latest information on currently circulating SARS-CoV-2 variant please see:

https://www.gov.uk/government/publications/investigation-of-novel-sars-cov-2-variant-variant-of-concern-20201201
https://www.who.int/en/activities/tracking-SARS-CoV-2-variants/

The mutation profiles of these variants and other circulating clades can be found at:
https://outbreak.info/situation-reports

In tube 2:

- Orf1ab region should be producing a fluorescence signal on FAM channel.
- IC should be producing a fluorescence signal on HEX/VIC channel.
- M region should be producing a fluorescence signal on ROX channel (see table below).

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FAM (Orf1a region)	HEX/VIC (IC)	ROX (M region)	Cy5 (N/A)	Results
Cq (+)	Cq (+)	Cq (+)	N/A	SARS-CoV-2 Positive
Cq (+)	Cq (+)	Cq (-)	N/A	SARS-CoV-2 Positive
Cq (-)	Cq (+)	Cq (+)	N/A	SARS-CoV-2 Positive
Cq (-)	Cq (+)	Cq (-)	N/A	SARS-CoV-2 Negative

Acceptance quality control criteria

Before data analysis, the following acceptance criteria for each run should be applied as an experimentation quality control:

- Any amplification in the Negative Control must be Cq>35 in all target channels for tube 1.
- Internal Control produces a positive amplification Cq<35 in the HEX/VIC channel only for tube 2 for each sample.
- Positive Control Tube 1 should give signal in all channels, FAM, VIC/HEX, ROX and CY5, at a Cq between 14-22.
- Positive Control Tube 2 should give signal in FAM and ROX at a Cq between 14-22.

9. Analytical performance

Introduction: In order to determine the analytical performance of Tube 1 of the EscapePLEX assay, SARS-CoV-2 whole genome RNA for three Variants of Concern (VOCs) from Twist Bioscience were used as targets (Table 1).

Table 1. Details of whole genome RNA of Variants of Concern used to test tube 1

Control	Variant of Concern	Present In	Country	GISAID Name	GenBank/ GISAID ID	Mutations	Channel
Control 16	Beta	B.1.351 lineage	South Africa	KRISP-EC-K005299/2020	EPI_ISL_678597	E484K	FAM
						K417N	HEX
Control 17	Gamma	P.1 lineage	Japan	IC-0564/2021	EPI_ISL_792683	E484K	FAM
						K417T	ROX
Control 18	Kappa	B.1.617.1 lineage	India	CT-ILSGS00361/2021	EPI_ISL_1662307	P681R	Cy5

To determine the performance of tube 2, wild-type (WT) SARS-CoV-2 whole genome RNA from Twist Bioscience was used. Unless otherwise stated, the following studies all utilised the Exsig™ Mag extraction system (Novacyt) to extract nucleic acids, using the KingFisher Flex system (ThermoFisher), with amplifications carried out using the Bio-Rad CFX Opus Real-Time PCR system.

9.1 Analytical sensitivity (Limit of detection)

9.1.1 Summary: The objective of this study was to determine the Analytical Sensitivity (Limit of Detection) of the SNPsig® SARS-CoV-2 (EscapePLEX) assay. The LoD is defined as the lowest concentration of analyte that could be reliably detected with 95% confidence. Three SARS-CoV-2 whole genome RNA Variants of Concern (VOCs) were used to represent the four mutations detected in Tube 1 in the EscapePLEX assay. The wild type (WT) SARS-CoV-2 whole genome RNA was used to represent WT SARS-CoV-2 detected in Tube 2.

The tentative LoD for Tube 1 and Tube 2 was determined by running 5 replicates across 5 contrivance levels (50, 20, 10, 5 and 1 copies per reaction) for all controls. The lowest concentration where all 5 replicates amplified was then verified in a larger scale study, with contrivance levels around the tentative LoD used. The LoD was established as the lowest concentration where all 20 replicates gave ≥95% positive amplification.

To verify the LoD of Tube 1, samples were run at three contrivance levels of controls 16, 17 and 18, with 20 replicates at 15, 20 and 25 copies/reaction in the final PCR reaction. In contrast, Tube 2 was run at four contrivance levels of the WT control with 20 replicates at: 3, 4, 5 and 6 copies/reaction in the final PCR reaction. The LoD of the overall tube was considered where all targets within each tube reached 95% confidence, i.e., E484K, K417N, K417T and P681R mutations, for tube 1, and ORF1ab and M gene targets in tube 2.

9.1.2 Results of the analytical sensitivity study are shown in Tables 2 and 3

Table 2. Verification LoD Data for Variants of Concern (Tube 1)

	Mean Conc (copies/µl)	Mean Conc (copies/rxn)	Total Replicates	Detection Rate (%)	Mean FAM Cq (STDV)	Mean HEX Cq (STDV)	Mean ROX Cq (STDEV)	Mean Cy5 Cq (STDV)
Control 16	1.25	25	20	20 (100)	34.90 (1.13)	32.17 (0.83)	N/A	N/A
	1.00	20	20	18 (90)	36.00 (1.37)	32.54 (0.67)	N/A	N/A
	0.75	15	20	20 (100)	36.43 (1.26)	33.57 (0.89)	N/A	N/A
Control 17	1.25	25	20	19 (95)	35.58 (0.85)	N/A	34.99 (0.58)	N/A
	1.00	20	20	17 (85)	36.13 (2.48)	N/A	34.77 (0.51)	N/A
	0.75	15	20	14 (70)	37.68 (1.56)	N/A	35.48 (0.76)	N/A
Control 18	1.25	25	20	19 (95)	N/A	N/A	N/A	35.16 (0.69)
	1.00	20	20	14 (70)	N/A	N/A	N/A	34.87 (0.62)
	0.75	15	20	15 (75)	N/A	N/A	N/A	36.00 (1.75)

Table 3. Verification LoD Data for WT SARS-CoV-2 (Tube 2)

Mean Conc (copies/µl)	Mean Conc (copies/rxn)	Total Replicates	Detection Rate (%)	Mean FAM Cq (STDV)	Mean HEX Cq (STDV)	Mean ROX Cq (STDV)
0.3	6	20	20 (100)	34.78 (0.52)	20.45 (0.52)	36.07 (1.03)
0.25	5	20	18 (90)	35.18 (0.86)	20.68 (0.52)	36.25 (0.89)
0.2	4	20	18 (90)	35.48 (0.82)	20.49 (0.50)	36.49 (1.12)
0.15	3	20	15 (75)	36.01 (1.06)	20.67 (0.56)	36.32 (1.14)

9.1.3 Conclusions: The data above demonstrates that the SNPsig® SARS-CoV-2 (EscapePLEX) assay tube 2 detects as little as 6 copies/reaction of SARS-CoV-2 whole viral genome RNA ≥95% of the time. Tube 1 detects as little as 25 copies/reaction of SARS-CoV-2 whole viral genome RNA containing the listed mutations ≥95% of the time.

9.2 Analytical specificity (Cross-reactivity)

9.2.1 Summary: The objective of this study is to assess the Analytical Specificity i.e., inclusivity and exclusivity for the SNPsig® SARS-CoV-2 (EscapePLEX) assay. This establishes whether the assay would detect all relevant strains and not detect non-targeted sequences. Analytical specificity (inclusivity) and 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® SARS-CoV-2 (EscapePLEX) assay. The *in-silico* analysis also evaluated assay inclusivity.

Three SARS-CoV-2 whole genome RNA Variants of Concern (VOC) from Twist Bioscience were used to represent the four mutations detected in Tube 1 of the SNPsig® SARS-CoV-2 EscapePLEX assay. Each was contrived to give a final concentration in the assay of 10,000 copies per µl. The Zeptometrix panel already contains a wild type SARS-CoV-2 RNA.

9.2.2 Results of the *in vitro* cross-reactivity testing are presented in Table 4 below:

Table 4: Specificity Results for SNPsig® SARS-CoV-2 EscapePLEX

Panel member	Tube 1				Tube 2		
	Average FAM Cq (E484K)	Average HEX Cq (K417N)	Average Rox Cq (K417T)	Average Cy5 Cq (P681R)	Average FAM Cq (ORF1ab)	Average HEX Cq (IEC)	Average Rox Cq (M)
Adenovirus 1	N/A	N/A	N/A	N/A	N/A	23.08	N/A
Adenovirus 3	N/A	N/A	N/A	N/A	N/A	23.28	N/A
Adenovirus 31	N/A	N/A	N/A	N/A	N/A	23.14	N/A
B. parapertussis	N/A	N/A	N/A	N/A	N/A	23.82	N/A
B. pertusis	N/A	N/A	N/A	N/A	N/A	23.51	N/A
C. pneumoniae	N/A	N/A	N/A	N/A	N/A	23.22	N/A
Coronavirus 229E	N/A	N/A	N/A	N/A	N/A	23.28	N/A
Coronavirus HKU-1	N/A	N/A	N/A	N/A	N/A	22.88	N/A
Coronavirus NL63	N/A	N/A	N/A	N/A	N/A	22.66	N/A
Coronavirus OC43	N/A	N/A	N/A	N/A	N/A	23.18	N/A
Influenza A H1N1pdm	N/A	N/A	N/A	N/A	N/A	23.07	N/A
Influenza AH1	N/A	N/A	N/A	N/A	N/A	22.86	N/A
Influenza AH3	N/A	N/A	N/A	N/A	38.51*	24.37	N/A
Influenza B	N/A	N/A	N/A	N/A	N/A	23.13	N/A
M. pneumoniae	N/A	N/A	N/A	N/A	N/A	22.82	N/A
Metapneumovirus 8	N/A	N/A	N/A	N/A	N/A	23.86	N/A
Parainfluenza 1	N/A	N/A	N/A	N/A	N/A	22.76	N/A
Parainfluenza 2	N/A	N/A	N/A	N/A	N/A	22.94	N/A
Parainfluenza 3	N/A	N/A	N/A	N/A	N/A	24.37	N/A
Parainfluenza 4	N/A	N/A	N/A	N/A	37.50*	24.55	N/A
Rhinovirus 1A	N/A	N/A	N/A	N/A	N/A	23.87	N/A
RSV A	N/A	N/A	N/A	N/A	N/A	23.79	N/A
SARS-CoV-2 (Wild type)	N/A	N/A	N/A	N/A	30.42	26.28	32.8
Panel Negative Control	N/A	N/A	N/A	N/A	N/A	29.64	N/A
SARS-CoV-2 Control 16	30.24	28.19	N/A	N/A	27.07	34.25	29.04
SARS-CoV-2 Control 17	31.2	N/A	29.7	N/A	27.25	30.32	29.66
SARS-CoV-2 Control 18	N/A	N/A	N/A	30.5	26.64	34.44	29.09
EscapePLEX Positive Control	14.52	13.11	14.12	14.00	17.08	N/A	17.31
NTC	N/A	N/A	N/A	N/A	N/A	N/A	N/A

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The red highlighted cells show Influenza AH3 and Parainfluenza 4 both had amplification in the FAM channel in one of the wells. As both samples were run in duplicate, and the other replication did not amplify, they were observed as potential contamination and were run again, in triplicate, alongside the negative control from the panel and SARS-CoV-2 (Wild type), see table 5 for details. Additional NTCs were run to determine if there was any contamination in the instrument. The re-run shows no amplification suggesting potential contamination of prior well.

Table 5: Specificity Results for Tube 2 Rerun Panel Samples

Panel member	Average FAM Cq (ORF1ab)	Average HEX Cq (IEC)
Influenza AH3	N/A	22.91
Parainfluenza 4	N/A	22.78
Panel Negative Control	N/A	27.42
SARS-CoV-2 (Wild type)	30.07	25.65

9.2.3 Conclusions: The results show that the SNPsig® SARS-CoV-2 EscapePLEX Assay exhibits no cross reactivity with any of the bacterial or viral nucleic acids tested from the NATtrol™ Respiratory Verification Panel. The samples from these panels are representative of true clinical samples, thus overall, this data confirms that the SNPsig® SARS-CoV-2 EscapePLEX assay maintains the expected inclusivity and exclusivity.

9.3 Precision (repeatability and reproducibility)

9.3.1 Summary: This study assesses the repeatability (intra-run) and reproducibility (inter-run) of the SNPsig® SARS-CoV-2 EscapePLEX assay. Precision was performed on three different batches when varying operators, instruments, and testing days. The study involved contriving SARS-CoV-2 negative saliva samples with a known copy number of synthetic RNA template representing the SARS-CoV-2 genomic region of interest: three SARS-CoV-2 whole genome RNAs were used to represent the four mutations detected in Tube 1: E484K, K417N, K417T and P681R; wild type SARS-CoV-2 whole genome RNA was used in Tube 2 of the assay.

Samples were contrived with three contrivance levels determined from the Limit of Detection (LoD) of the assay, reported in the Analytical Sensitivity study. The samples were extracted using the KingFisher™ Flex extraction platform with the exsig® Mag Extraction System and for this study, the confirmed LoD of 25 copies/rxn for tube 1 and a tentative LoD of 5 copies/rxn was used for tube 2.

Variance was assessed from operators, instruments, and day of testing. Two different operators performed the study over two days with two Bio-Rad CFX Opus Real-Time PCR instruments. A total of 10 replicates were obtained for each contrivance level. The precision was measured by reporting the % Coefficient of Variance. A CV lower than 10% was considered as desirable and a CV lower than 15% was considered as acceptable.

9.3.2 Results of the precision studies are shown in Tables 6 to 11, with CV values of greater than or equal to 10% are highlighted.

Table 6: Coefficient of variance (%) for SNPsig® SARS-CoV-2 EscapePLEX assay Tube 1/ Batch 1

Contrivance level	Channel	Inter operator	Inter machine	Inter day	Intra run
15x LoD	FAM	5.06	6.15	4.01	3.89
	HEX	5.93	7.78	5.48	5.90
	ROX	7.57	7.74	6.79	5.71
	Cy5	3.52	3.78	2.97	3.38
10x LoD	FAM	5.85	4.66	8.00	3.19
	HEX	7.45	7.44	7.38	6.14
	ROX	5.93	7.09	5.54	5.53
	Cy5	6.96	6.32	5.91	8.79
5x LoD	FAM	4.59	4.08	3.88	3.67
	HEX	4.54	3.54	3.51	3.86
	ROX	4.12	4.05	4.71	4.55
	Cy5	2.55	3.10	1.81	2.11

Table 7: Coefficient of variance (%) for SNPsig® SARS-CoV-2 EscapePLEX assay Tube 1/ Batch 2

Contrivance level	Channel	Inter operator	Inter machine	Inter day	Intra run
15x LoD	FAM	7.85	6.21	8.93	6.80
	HEX	7.51	7.34	8.68	7.37
	ROX	7.75	6.43	7.92	5.99
	Cy5	5.87	2.65	2.67	1.79
10x LoD	FAM	4.97	4.83	4.28	5.72
	HEX	7.57	5.01	5.50	6.03
	ROX	4.61	4.50	4.77	5.40
	Cy5	4.77	6.15	5.89	5.11
5x LoD	FAM	4.85	5.38	7.57	5.98
	HEX	5.15	4.01	5.09	4.59
	ROX	4.70	4.45	5.78	4.24
	Cy5	3.35	3.04	2.52	2.04

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Table 8: Coefficient of variance (%) for SNPsig® SARS-CoV-2 EscapePLEX assay Tube 1/ Batch 3

Contrivance level	Channel	Inter operator	Inter machine	Inter day	Intra run
15x LoD	FAM	5.97	2.99	1.67	4.18
	HEX	2.37	2.45	2.33	4.29
	ROX	1.88	0.51	1.49	4.85
	Cy5	0.46	1.86	1.61	0.43
10x LoD	FAM	5.14	5.77	1.56	5.25
	HEX	1.79	5.91	1.67	3.54
	ROX	1.92	4.82	2.12	5.18
	Cy5	1.43	2.15	2.76	1.18
5x LoD	FAM	6.78	2.80	2.87	3.78
	HEX	2.88	2.44	3.90	2.67
	ROX	2.67	1.20	1.27	4.78
	Cy5	1.22	2.25	1.68	1.27

Table 9: Coefficient of variance (%) for SNPsig® SARS-CoV-2 EscapePLEX assay Tube 2/Batch 1

Contrivance level	Channel	Inter operator	Inter machine	Inter day	Intra run
15x LoD	FAM	8.39	8.74	12.41	8.80
	HEX	3.52	1.07	4.31	1.07
	ROX	9.55	10.53	12.07	10.73
10x LoD	FAM	6.60	6.92	9.36	7.10
	HEX	3.50	0.97	5.15	0.86
	ROX	8.25	9.10	9.66	9.76
5x LoD	FAM	4.68	5.16	12.20	4.99
	HEX	3.29	0.95	4.62	0.99
	ROX	5.54	6.81	9.86	6.12

Table 10. Coefficient of variance (%) for SNPsig® SARS-CoV-2 EscapePLEX assay Tube 2/Batch 2

Contrivance level	Channel	Inter operator	Inter machine	Inter day	Intra run
15x LoD	FAM	7.98	8.34	10.77	8.26
	HEX	3.57	1.97	5.05	1.03
	ROX	8.97	9.84	10.74	9.70
10x LoD	FAM	6.26	6.81	9.27	6.78
	HEX	3.63	2.22	4.66	0.74
	ROX	7.51	8.49	7.53	8.13
5x LoD	FAM	4.64	4.87	9.61	4.73
	HEX	3.82	2.58	4.79	2.47
	ROX	5.85	6.42	7.93	6.19

Table 11: Coefficient of variance (%) for SNPsig® SARS-CoV-2 EscapePLEX assay Tube 2/Batch 3

Contrivance level	Channel	Inter operator	Inter machine	Inter day	Intra run
15x LoD	FAM	5.66	4.15	10.59	4.18
	HEX	4.23	3.82	4.13	4.29
	ROX	5.30	5.54	8.33	4.85
10x LoD	FAM	4.90	4.95	8.64	5.25
	HEX	4.67	3.46	4.00	3.54
	ROX	6.01	4.97	7.59	5.18
5x LoD	FAM	3.76	4.41	8.28	3.78
	HEX	3.87	2.77	2.81	2.67
	ROX	4.99	4.81	6.91	4.78

9.3.3 Conclusions: The results show that the EscapePLEX kit produces reproducible and repeatable results across all channels. The Cq variance produced within a run and between operators, machines, and days is of an acceptable level as all coefficients of variance are below 15%. Most of the coefficients of variance are below 10%, indicating that precision of the kit is desirable in most cases. The comparison with the most CV values above 10% was inter-day. Since the contrived samples used were prepared at the beginning of a day and used for every plate on that day, the inter-day variance could be explained by manual differences by operators rather than being reflective of the precision of the EscapePLEX kit.

9.4 Accuracy (trueness and precision)

9.4.1 Summary: Diagnostic accuracy of the SNPsig® SARS-CoV-2 (EscapePLEX) assay was determined by generating a Positive Percentage Agreement (PPA), Negative Percentage Agreement (NPA) and Overall Percentage Agreement (OPA). Percentage Agreement is generated by conducting blind randomised testing using the EscapePLEX assay and comparing it to the contrivance status of 180 total samples, contrived at 5x the LoD of the assay: 30 contrived with Control 16 (assayed with Tube 1), 30 contrived with Control 17 (Tube 1), 30 contrived with Control 18 (Tube 1), 30 negative (Tube 1), 30 contrived with Wildtype (assayed with Tube 2) and 30 negative (Tube 2). The positive, negative, and overall percentage agreement (PPA, NPA, OPA) was calculated by comparing test results with the recorded contrivance status for each mutation or the wild-type virus (each detected in a separate channel).

9.4.2 Results are summarised in terms of the PPA, NPA and OPA in Table 12.

Table 12. Summary of percentage agreements and 95% confidence intervals.

Tube/Control	Agreement	Level	95% CI (%)
Tube 1: FAM	PPA	88.52	93.89-100
	NPA	100.00	N/A
	OPA	94.17	88.45-97.15
Tube 1: HEX	PPA	96.67	93.97-99.80
	NPA	98.89	83.33-99.41
	OPA	98.33	94.13-99.54
Tube 1: ROX	PPA	100.00	N/A
	NPA	100.00	N/A
	OPA	100.00	N/A
Tube 1: Cy5	PPA	96.67	95.91-100
	NPA	100.00	N/A
	OPA	99.17	95.43-99.85
Tube 2	PPA	100.00	N/A
	NPA	96.67	88.65-100
	OPA	98.33	91.15-99.71

9.4.3 Conclusions The results show that the Path-SNPsig® SARS-CoV-2 EscapePLEX assay met the performance criteria in detecting each mutuation or wild-type virus as the OPA was ≥ 90% for every target.

9.5 Interfering Substances

9.5.1 Summary: The objective of this study is to evaluate the effects of potential exogenous and endogenous interfering substances present within target saliva samples on the performance of the SNPsig® SARS-CoV-2 EscapePLEX assay. Changes in performance of the assay were analysed by Cq values of samples containing the potential interfering substances. Where an interferent effect was observed with any of the substances tested, a further dose-response study was performed to establish to what extent the interferent can be seen to cause a change. Each interfering substance was run using different control RNA/tube combinations as set out below.

- Twist SARS-CoV-2 Control 16 with Tube 1
- Twist SARS-CoV-2 Control 17 with Tube 1
- Twist SARS-CoV-2 Control 18 with Tube 1
- Twist SARS-CoV-2 WT with Tube 2

Each control RNA was contrived at 5x the LoD of Path-SNPsig SARS-CoV-2 EscapePLEX assay, as determined by the Analytical Sensitivity study. After amplification, each substance was compared to its control and given a pass/fail based on the acceptance criteria (a change in Mean Cq of +/1.5 versus the control substance, or a t-test between the interfering substance and its control produces a P-value <0.05).

9.5.2 Results of the screening of interferents are shown in Tables 13 and 14

Table 13. Interference screen results for Control 16, 17, 18 and WT:

Substance	Interference screen results for:						
	Control 16 FAM (tube 1)	Control 16 HEX (tube 1)	Control 17 FAM (tube 1)	Control 17 ROX (tube 1)	Control 18 Cy5 (tube 1)	WT control FAM (tube 2)	WT Control ROX (tube 2)
Blood	Fail	Pass	Pass	Pass	Pass	Pass	Pass
Nasacort	Pass	Pass	Pass	Pass	Pass	Pass	Pass
Dymista	Pass	Pass	Pass	Pass	Pass	Pass	Pass
Oseltamivir	Pass	Pass	Pass	Pass	Pass	Pass	Pass
Tobramycin	Pass	Pass	Pass	Pass	Pass	Pass	Pass
Strepsils	Pass	Pass	Pass	Pass	Pass	Pass	Pass
Amylase	Fail	Pass	Pass	Pass	Pass	Pass	Pass
Oxymetazoline	Fail	Pass	Pass	Pass	Pass	Pass	Pass
Dexamethasone	Pass	Pass	Pass	Pass	Pass	Pass	Pass
Fluticasone	Pass	Pass	Pass	Pass	Pass	Pass	Pass
Guaifenesin	Pass	Pass	Pass	Pass	Pass	Pass	Pass
Mupirocin	Pass	Pass	Pass	Pass	Pass	Pass	Pass
Mucin	Pass	Pass	Pass	Pass	Pass	Pass	Pass

Table 14. Dose response results for control 16 using different Amylase/Blood/Oxymetazoline concentrations in the FAM and HEX channels

Substance	FAM Channel				HEX Channel			
	x Testing concentration	Concentration	Mean Cq	Result	x Testing concentration	Concentration	Mean Cq	Result
Amylase g/ml	0.10	0.792	32.73	Pass	0.10	0.792	28.89	Pass
	0.25	1.980	34.29	Pass	0.25	1.980	29.03	Pass
	0.50	3.960	31.29	Pass	0.50	3.960	29.07	Pass
	1.00	7.920	31.85	Pass	1.00	7.920	29.26	Pass
Blood g/ml	0.10	0.00076	32.81	Pass	0.10	0.00076	28.44	Pass
	0.25	0.00190	32.64	Pass	0.25	0.00190	29.16	Pass
	0.50	0.00380	32.39	Pass	0.50	0.00380	28.83	Pass
	1.00	0.00760	32.15	Pass	1.00	0.00760	28.96	Pass
Oxymetazoline ng/ml	0.10	0.06	33.15	Pass	0.10	0.06	29.31	Pass
	0.25	0.15	32.18	Pass	0.25	0.15	29.20	Pass
	0.50	0.30	32.10	Pass	0.50	0.30	28.84	Pass
	1.00	0.60	33.69	Pass	1.00	0.60	29.23	Pass

9.5.3 Conclusions: The results showed that amylase, blood and oxymetazoline displayed interference in the FAM channel of control 16 during the initial interference screen. All other substances passed the acceptance criteria for all controls in all relevant channels and therefore were deemed not to have interfered with the assay. Amylase, blood and oxymetazoline were further tested against a dose response analysis using control 16/FAM channel, which showed that these now passed the initial acceptance criteria at all concentrations tested. It can be determined from the interference screen and subsequent dose response analysis

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that none of the substances listed caused any interference with the SNPsig® SARS-CoV-2 EscapePLEX assay.

9.6 Alternative Instrument Testing

9.6.1 Summary: The objective of these studies was to verify the limit of detection (LoD) of the SNPsig® SARS-CoV-2 EscapePLEX assay on alternative real-time PCR platforms: the genesig® q32 Real-time PCR platform; the Applied Biosystems 7500 Fast Real-Time PCR system; and the Roche® Lightcycler 480 II. All these instruments are capable of reading in the FAM, HEX, ROX and Cy5 channels, and are therefore compatible with the assay. The 7500 Fast system was used in "ABI 7500" mode, which equates to the standard ABI 7500 instrument without fast cycling capabilities.

Saliva samples were contrived at the LoD determined on the Bio-Rad CFX Opus platform, and also at contrivance levels above and below the LoD, using the three Variant of Concern controls, or the WT SARS-CoV-2 RNA. 20 replicates of each contrivance level were tested, with a >95% call rate in each of the relevant channels required for verification of LoD.

9.6.2: Results for the different real-time PCR systems are shown in Tables 15 to17

Table 15. Limit of detection study for the genesig® q32 Real-Time PCR Platform

Control	Tube	Contrivance (copies/rxn)	Mean Cq (FAM)	Mean Cq (HEX)	Mean Cq (ROX)	Mean Cq (Cy5)	Call Rate
16	1	30	34.27	32.78	N/A	N/A	20/20
16	1	25	35.33	32.83	N/A	N/A	19/20
16	1	20	35.29	33.34	N/A	N/A	19/20
17	1	30	36.70	N/A	34.10	N/A	19/20
17	1	25	36.67	N/A	34.52	N/A	19/20
17	1	20	37.38	N/A	34.78	N/A	14/20
18	1	30	N/A	N/A	N/A	33.35	20/20
18	1	25	N/A	N/A	N/A	33.06	20/20
18	1	20	N/A	N/A	N/A	34.79	20/20
WT	2	6	32.73	20.68	33.44	N/A	20/20
WT	2	5	32.79	20.71	33.49	N/A	20/20
WT	2	4	32.75	20.80	33.27	N/A	20/20

Table 16. Limit of detection study for the Applied Biosystems 7500 Fast system

Control	Tube	Contrivance (copies/rxn)	Mean Cq (FAM)	Mean Cq (HEX)	Mean Cq (ROX)	Mean Cq (Cy5)	Call Rate
16	1	30	35.94	34.44	N/A	N/A	19/20
16	1	25	35.71	33.83	N/A	N/A	19/20
16	1	20	36.08	34.28	N/A	N/A	18/20
17	1	30	36.46	N/A	30.89	N/A	20/20
17	1	25	36.95	N/A	28.15	N/A	20/20
17	1	20	34.97	N/A	25.99	N/A	20/20
18	1	30	N/A	N/A	N/A	34.91	20/20
18	1	25	N/A	N/A	N/A	35.93	20/20
18	1	20	N/A	N/A	N/A	36.08	17/20
WT	2	6	33.71	22.04	34.57	N/A	20/20
WT	2	5	34.00	22.09	34.40	N/A	20/20
WT	2	4	33.82	22.15	34.29	N/A	20/20

Table 17. Limit of detection study for the Roche® Lightcycler 480 II

Control	Tube	Contrivance (copies/rxn)	Mean Cq (FAM)	Mean Cq (HEX)	Mean Cq (ROX)	Mean Cq (Cy5)	Call Rate
16	1	25	35.32	32.99	N/A	N/A	20/20
16	1	20	38.19	37.11	N/A	N/A	13/20
16	1	15	38.64	37.1	N/A	N/A	2/20
17	1	35	36.42	N/A	34.28	N/A	20/20
17	1	30	36.41	N/A	34.47	N/A	20/20
17	1	25	36.54	N/A	34.39	N/A	20/20
18	1	35	N/A	N/A	N/A	33.38	20/20
18	1	30	N/A	N/A	N/A	35.2	20/20
18	1	25	N/A	N/A	N/A	35.6	13/20
WT	2	6	33.93	35.57	19.96	N/A	20/20
WT	2	5	33.06	34.50	19.88	N/A	20/20
WT	2	4	33.62	34.46	21.72	N/A	12/20

9.6.3 Conclusions: The LoD of 25 copies/reaction for tube 1 and 6 copies for tube 2 (verified through the CFX Opus Real-Time PCR System) was confirmed on all alternative platforms, with the exception of the LightCycler, which instead achieved a call rate >95% when 30 copies/reaction of SARS-CoV-2 control 18 RNA were present in tube 1.

10. Clinical Performance

A multi-site clinical performance study was performed at the Molecular Diagnostics Lab in University of Birmingham's Department of Clinical Immunology Service in Birmingham, and Whiston Hospital in Prescot.

A total of 24 known extracted RNA SARS-CoV-2 positive clinical samples were run with SNPsig SARS-CoV-2 EscapePLEX following instructions provided by Primerdesign™. To verify the correct identification of the four mutations in the samples, all samples were sequenced in parallel. Sequencing results only provided the variant detected, not mutation status at individual target sites, therefore the study at the level of mutations was done following the mutation profiles per variant available at outbreak.info (https://outbreak.info/situation-reports). All samples were confirmed as SARS-CoV-2 positive samples by SNPsig SARS-CoV-2 EscapePLEX tube 2.

The status of the sample was defined as “true positive”/”true negative” when sequencing data agreed with the results obtained with SNPsig SARS-CoV-2 EscapePLEX and considered “false positive”/”false negative” when in disagreement.

		Sequencing results		
		Positive	Negative	Total
E484K (FAM)	Positive	2	1	3
	Negative	0	21	21
	Total	2	22	24

		Sequencing results		
		Positive	Negative	Total
K417N (HEX)	Positive	2	0	0
	Negative	0	22	24
	Total	0	24	24

		Sequencing results		
		Positive	Negative	Total
P681R (Cy5)	Positive	13	0	13
	Negative	1	10	11
	Total	14	10	24

The diagnostic sensitivity and specificity for each mutation can be seen in the table below.

	E484K	K417N	K417T	P681R
Clinical sensitivity	100.0%	100.0%	N/I	92.9%
Clinical specificity	95.5%	100.0%	100.0%	100.0%

No positive results were obtained for mutation K417T due to a lack of SARS-CoV-2 positive cases carrying such a mutation at the sites involved in the study, and therefore, the clinical sensitivity could not be determined.

11. Disposal

Dispose of unused kit reagents, human specimens and laboratory clinical waste according to national regulations. Refer to Section 7 for guidance weblinks

12. Manufacturer

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Symbols	Meanings
	CE Mark
	EU Authorized Representative
	Batch Code
	In Vitro Diagnostics
	Keep away from sunlight (primer/probe mix)
	Catalogue number
	Consult Electronic Instructions for Use
	Manufacturer
	Positive Control
	Use by Date
	Single Use Symbol

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