

Instruction for Use

Winterplex (genesig® Real-Time PCR
SARS-CoV-2 Winterplex
assay)
96 tests

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1. Intended purpose

The genesig® Real Time PCR SARS-COV-2 Winterplex is a CE marked, *in vitro* diagnostic, real-time, reverse transcriptase PCR (RT-PCR) multiplex assay intended for the qualitative detection of nucleic acid from SARS-CoV-2 (ORF1ab, S and M gene targets), Influenza A (Flu A), Influenza B (Flu B) and Respiratory Syncytial Virus (RSV A and B) in nasopharyngeal swabs, oropharyngeal swabs and sputum specimens. This multiplex assay provides rapid screening of individuals suspected of SARS-CoV-2, Flu A, Flu B and RSV infections and aids the diagnosis of suspected disease in patients.

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

The genesig® Real Time PCR SARS-COV-2 Winterplex 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 extraction systems and the designated PCR platforms listed in Section 8.

Positive results are indicative of the presence of SARS-CoV-2, Flu A, Flu B or RSV RNA. Positive results do not rule out co-infection with other bacteria or other viruses. Negative results do not preclude SARS-CoV-2, Flu A, Flu B or RSV infections 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 genesig® Real Time PCR SARS-COV-2 Winterplex assay. This time includes the time to extract nucleic acid from a specimen, PCR set-up, PCR run time, and to availability of results.

2. Summary

The genesig® Real Time PCR SARS-COV-2 Winterplex is a CE marked, *in vitro* diagnostic, real-time, reverse transcriptase PCR (RT-PCR) multiplex assay intended for the qualitative detection of nucleic acid from SARS-CoV-2 (ORF1ab, S and M gene targets), Influenza A (Flu A), Influenza B (Flu B) and Respiratory Syncytial Virus (RSV A and B) in nasopharyngeal swabs, oropharyngeal swabs and sputum specimens.

This multiplex assay provides combined rapid screening of individuals suspected of SARS-CoV-2, Flu A, Flu B and RSV infections and aids the diagnosis of suspected disease in patients. Specimen test results are available to interpret in under three hours using the genesig® Real Time PCR SARS-COV-2 Winterplex assay.

The genesig® Real Time PCR SARS-COV-2 Winterplex assay is intended for use by qualified and trained clinical laboratory personnel.

Positive results are indicative of the presence of SARS-CoV-2, Flu A, Flu B or RSV RNA. Negative results do not preclude SARS-CoV-2, Flu A, Flu B or RSV infections. Positive and Negative results must be combined with clinical observations, patient history, and epidemiological information.

3. Test principle

The genesig® SARS-COV-2 Winterplex Real Time PCR assay is an *in vitro* diagnostic test based on the standard hydrolysis probe system known as TaqMan® Technology. Real-Time PCR technology utilizes polymerase chain reaction (PCR) for the amplification of specific target sequences and target specific probes for the detection of the amplified RNA. The probes are labelled with fluorescent reporter and quencher dyes.

RNA isolated and purified from nasopharyngeal or oropharyngeal swabs is reverse transcribed to cDNA and subsequently amplified using a validated Real-Time PCR instrument: genesig® q32 Real-Time PCR instrument, or Bio-Rad CFX Opus Real-time PCR Detection System (Maestro software 2.0, version 5.0). During PCR cycling, the probe anneals to a specific target sequence located between the forward and reverse primers. The probe is cleaved by the 5' nuclease activity of the Taq polymerase during the extension phase of the PCR cycle, causing the reporter dye to separate from the quencher dye, generating a fluorescent signal. With each PCR cycle, additional reporter dye molecules are released from the probe, increasing the fluorescence intensity. Fluorescence intensity is recorded at each cycle of the PCR by the Real-Time PCR machine.

The assay consists of labelled probes in one test specific for SARS-CoV-2 (ORF1ab, S and M gene targets) in FAM, Cy5 and ROX channels, respectively; and a second parallel test to detect Influenza A (Flu A), Influenza B (Flu B) and Respiratory Syncytial Virus (RSV A and B) using FAM, Cy5 and ROX fluorophores respectively.

The assay includes an internal extraction control (genesig® Easy RNA Internal Extraction control), which is added to the IVD nucleic acid extraction system (not provided) to measure RNA extraction purity, detect PCR inhibition and confirm the integrity of the PCR run.

The probe to detect the internal extraction control (which is from a non-biologically relevant exogenous source) is present in both tests and is labelled with the HEX fluorophore.

4. Materials

The genesig® Real-Time PCR SARS-COV-2 Winterplex assay contains:

Reagent label	Number of Vials 96 tests	Volume (µl per vial)	Lid colour	Resuspended with
Onestep Lyophilised Master Mix	4	525*	Gold, vial stored in sealed foil pouch	Master Mix Resuspension Buffer
ORF1ab/S/M Primer & Probe Mix (including IEC primer/probe mix)	1	220*	Amber, vial stored in sealed foil pouch	Template preparation buffer
FluA/FluB/R SV Primer & Probe Mix (including IEC primer/probe mix)	1	220*	Yellow (Amber body), vial stored in sealed foil pouch	Template preparation buffer
Master Mix Resuspension Buffer	4	750	Blue	n/a
Template preparation buffer	3	1500	Yellow	
Water RNase/DNase Free	1	1500	White	
genesig® Winterplex Positive control template	1	800*	Red, vial stored in sealed foil pouch	Template preparation buffer
genesig® Winterplex Internal extraction control (IEC)	2	1000*	Blue, vial stored in sealed foil pouch	

*The projected volume once resuspended

The SARS-COV-2 Winterplex Primer & Probe Mix consists of two tubes. ORF1ab/S/M primer & probe tube contains the primers and FAM labelled probe specific to the ORF1ab region of SARS-CoV-2, the primers and Cy5 labeled probe specific to the S gene of SARS-CoV-2, and the primers and

ROX labelled probe specific to the M gene of SARS-CoV-2, as well as the primers and HEX labelled probe for the genesig® Winterplex RNA Internal extraction control (IEC). FluA/FluB/RSV primer & probe tube contains the primers and FAM labeled probe for the detection of Influenza A, primers and Cy5 labelled probe specific to Influenza B, primers and ROX labeled probe for RSV, and the primers and HEX labelled probe for the genesig® Winterplex RNA IEC.

The OneStep Lyophilised Master Mix, the two Primers & Probes mixes, genesig® Winterplex Positive control template and genesig® Winterplex RNA IEC are all provided lyophilised. The table above indicates which buffer to use, as well as the volume to add, to resuspend these reagents.

5. Storage

Storage conditions

- The genesig® Real-Time PCR SARS-COV-2 Winterplex assay is shipped at ambient temperatures but must be stored at -20°C upon arrival.
- The genesig® Real-Time PCR SARS-COV-2 Winterplex 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 genesig® Real-Time PCR SARS-COV-2 Winterplex 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) 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 genesig® Real-Time PCR SARS-COV-2 Winterplex 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 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.

Storing Specimens

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

8. Assay procedure

Reagent and Controls Preparation

- OneStep Lyophilised Master Mix preparation
- Upon receipt, the dried master mix can be stored at -20°C. Do not use after the expiry date (see product label).
- Using aseptic technique, resuspend in 525 µl of Master mix resuspension buffer, gently swirl to mix.
- Store at -20°C. Resuspended master mix is stable for one month when stored at -20°C.
- Freeze/ thaw cycles should be minimized and not exceed 5 freeze/thaws. The reagent once resuspended can be aliquoted into smaller volumes if required and stored at -20°C.

SARS-COV-2 Winterplex and IEC Primer/Probe mix preparation

- Two primer/probe mixes are provided, ORF1ab/S/M primer probe mix and FluA/FluB/RSV primer probe mix. Both mixes also include primers and probe for the detection of the IEC.
- Upon receipt, the dried primers/probes mix can be stored at -20°C. Do not use after the expiry date (see product label).
- Precautions: this reagent should only be handled in a clean area and not exposed to light.
- Using aseptic technique, resuspend each dried primer/probe mix in 220 µl of Template preparation buffer and vortex to mix.
- Store at -20°C. Resuspended primer/probe mix is stable for one month when stored at -20°C.
- Freeze/ thaw cycles should be minimized 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 exposed sunlight.

genesig® SARS-COV-2 Winterplex Positive control template preparation

- The genesig® SARS-COV-2 Winterplex Positive control template (PCT) is provided in a sealed foil envelope and contains a mixture of high copy

number synthetic DNA templates and should be opened and processed away from clinical specimens and kit components to avoid cross-contamination.

- The PCT tube contains synthetic DNA representing the genomic regions of interest for the assay targets of: ORF1ab for SARS-CoV-2, S gene of SARS-CoV-2, M gene of SARS-CoV-2, Influenza A, Influenza B and RSV. Following resuspension, this will be at a concentration of 1.25×10^5 copies per μl .
- Caution: This reagent contains a high copy number of positive control material and should be handled with caution in a dedicated nucleic acid handling area to prevent possible contamination of other kit reagents and clinical specimens.
- Upon receipt, the dried PCT can be stored at -20°C . Do not use after the expiry date (see product label).
- Using aseptic technique, resuspend the dried PCT in 800 μl of Template preparation buffer, vortex thoroughly. Resuspended PCT is stable for one month when stored at -20°C .
- Freeze/ thaw cycles should be minimized and not exceed 5 freeze/thaws. The reagent once resuspended can be aliquoted into smaller volumes if required and stored at -20°C .
- To ensure PCR run validity, the PCT should produce amplification in the FAM channel for the ORF1ab (SARS-CoV-2) and Influenza A targets, amplification in the Cy5 channel for the S gene (SARS-CoV-2) and Influenza B targets, and amplification on the ROX channel for the M gene (SARS-CoV-2) and RSV.

genesig® Winterplex RNA Internal extraction control (IEC) preparation

- The genesig® Winterplex RNA Internal extraction control (IEC) 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 IEC can be stored at -20°C . Do not use after the expiry date (see product label).
- Using aseptic technique, resuspend the dried IEC in 1000 μl of Template preparation buffer, vortex thoroughly. Resuspended IEC is stable for one month when stored at -20°C .
- Freeze/ thaw cycles should be minimized and not exceed 5 freeze/thaws. The reagent once

resuspended can be aliquoted into smaller volumes if required and stored at -20°C .

Negative Extraction Control (NEC) preparation

- Prepare at least 1 negative extraction control (NEC) every time RNA is extracted from a cohort of samples. In addition, each PCR run should include a minimum of 1 NEC, prepared in parallel with the clinical samples.
- The NEC is an extraction with no clinical specimen/sample added, it is prepared by extracting from RNase/DNase free water. The IEC is added to the NEC sample during extraction as directed by the manufacturer's IFU. This NEC will serve as the negative control for the entire testing system and to check for contamination during PCR plate set-up.

No Template Control

- DNase/RNase free water is provided to use as a no template control (NTC), if required, in addition to the NEC (refer to Section 6.5)
- The NTC is used to check for contamination during PCR plate set-up.

General Preparation

Equipment 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 RNA/DNA remover to minimize the risk of nucleic acid contamination.
- Performance of the genesig® Real-Time PCR SARS-COV-2 Winterplex assay is dependent upon the amount and quality of RNA purified from human specimens. The following commercially available RNA extraction kits and procedures have been validated for recovery and purity of RNA for use with this assay:
- Automated extraction system GenoXtract® from Bruker HAIN Lifescience GmbH using the GXT NA Extraction kit.
- Qiagen extraction system with QIAamp® Viral RNA Mini kit (Qiagen, Germany)
- exsig™ Mag extraction kit from Primerdesign Ltd.
- Please consult the manufacturer's IFU of the chosen extraction system for full usage details.

Assay Set Up

Sample Preparation Procedure

Nasopharyngeal swabs, oropharyngeal swabs, saliva	
Collection	Swabs: Dacron or polyester flocked swabs in viral transport medium Saliva: Viral transport medium in sterile container
Transport temperature*	2-8°C ≤ 72 hrs
Short-term storage (pre-extraction)*	2-8°C ≤ 72 hrs
Long-term storage (pre-extraction)*	≤ -70°C for longer periods
Extraction sample volume	550 µL**
Extraction elution volume	50 µL

*These are CDC recommendations: CDC 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>

**Sample refers to the viral transport medium provided in the sample container serving as the repository for the swab.

Extraction sample volume and extraction elution volume as recommended for the GXT NA (CE) on the HAIN GenoXtract® from Bruker.

RNA extraction

The results of the genesis® Real-Time PCR SARS-COV-2 Winterplex assay is dependent upon the amount and quality of template RNA purified from human specimens.

- Consult the IFU of the extraction system for full usage details.
- Prepare at least 1 negative extraction control (NEC) each time a cohort of samples are processed for RNA extraction (i.e. an extraction with no clinical specimen/sample added).
- The genesis® Winterplex RNA Internal extraction control (IEC) should be resuspended in 1000 µl template preparation buffer. It should be incorporated in the extraction as directed by the extraction system IFU. Primerdesign recommends 20 µl is added per sample, directly into the lysis stage of the extraction.
- **The internal extraction control should not be added directly to the clinical specimen/ sample**

before RNA extraction (i.e. not before the clinical specimen/sample is mixed with a lysis buffer of the nucleic acid extraction kit/system). Doing so may compromise the testing.

- Where the IFU provides no specific guidance for the addition of an Internal extraction control or where an automated system does not support the addition of 20 µl IEC, please contact Primerdesign for guidance.

Master Mix Setup

Each of the two primer/probe tubes (ORF1ab/S/M and FluA/FluB/RSV) in this assay must be treated separately for plate setup, i.e. each patient sample, PTC, NTC and NEC must be tested against each primer/ probe mix. Therefore, follow the below master mix setup instructions for each of the two tubes.

- Resuspend each of the two primer/probe tubes in 220 µl of template preparation buffer, vortex to mix.
- Resuspend the OneStep Lyophilised Master Mix in 525 µl Master Mix Resuspension Buffer, gently swirl to mix.
- Plate set-up configuration can vary with the number of specimens. An NEC must be included in each plate set-up (refer to Section 6.5 and 8.2 on how to prepare NEC). NTCs should be included in each plate set-up. A PCT must be included in each plate set-up (note: one positive well is sufficient for all targets for simultaneous detection on multiple channels)
 - 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 NEC, NTC and any clinical specimen/ samples.
 - This is to prevent plate set-up, reagent, or specimen contamination with the PCT.
- Determine the number of reactions (n) to set up per assay (including NEC, PCT and any NTCs 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
- Prepare two reaction mixes, one for each primer/probe mix (ORF1ab/S/M and FluA/FluB/RSV). Label two 1.5ml DNase/RNase free

tubes (for example, COVID-19 and Flu/RSV). Dispense the following resuspended components into the correct labelled tube:

Reaction mix Component	1 x volume required (µl)*
Onestep Lyophilised Master Mix	10*
Primer & Probe mix (either ORF1ab/S or FluA/FluB/RSV)	2*

*Multiply all numbers by (N). Refer to step (d) above, to ensure there is sufficient reaction mix for all samples, NEC, PCT and NTCs to be tested.

- f) Add 12 µl of the ORF1ab/S/M reaction mix into the number of wells required for your testing, in an appropriate 96 well plate for your chosen PCR platform. Include 1 well for the PCT, 1 well for the NEC and 1 well for the NTC for each PCR plate.
- g) Add 12 µl of the FluA/FluB/RSV reaction mix into empty wells of the PCR plate or a second PCR plate, according to your plate setup. Include 1 well for the PCT, 1 well for the NEC and 1 well for the NTC for each primer/probe reaction mix.
- h) Add 8 µl of the following into the appropriate wells according to your plate setup:
 - a. NEC (please refer to Section 8 - Reagent and Controls Preparation)
 - b. NTC (please refer to Section 8 - Reagent and Controls Preparation)
- 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 8 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 8 µl of PCT (please refer to Sections 6.3) into the appropriate wells according to your plate set up. Seal the plate with an appropriate seal and place in the instrument.

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

- CFX Touch™ (Bio-Rad) & CFX Opus™ (Bio-Rad) Real-Time PCR Detection System Instrument Guide (as per Bio-Rad Laboratories Inc. Manual (2017)) *Dreskin EJ, Lowery J, Baker J, Nguyen N. A model qPCR Instrument Comparability Study: CFX96 Touch and CFX Opus 96 Real-Time PCR Systems. Bio-Rad Laboratories Inc. 2000*
- genesig® q32 Instrument Guide (2020) software version 3.5.21

a) Enter the following amplification program:

Steps	Time	Temperature	Cycles
Reverse Transcription	10 min	55°C	1
Initial Denaturation (Taq Activation)	2 min	95°C	1
Denaturation	10 sec	95°C	45
Annealing and Extension	60 sec	60°C*	

*Acquisition must be performed at the end of this stage, fluorogenic data should be collected through the **FAM**, **HEX/VIC**, **ROX** and **Cy5** channels.

- b) Ensure wells loaded with clinical sample(s) are designated as "Sample Type – Unknown"
- c) Ensure the well loaded with PCT is designated as "Sample Type – Positive Control"

Interpretation of Results

Acceptance criteria of controls included in the genesig® Real-Time PCR SARS- COV-2 Winterplex 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:

- a) NEC is free from amplification in the FAM (465-510), Cy5 (618-660) and ROX (575-610) channel, and that NEC produces positive amplification in the VIC/HEX (533-580) channel (this is detection of the genesig® Winterplex RNA Internal Extraction control).
- b) PCT produces a Cq of between 14-22 in the FAM (465-510), Cy5 (618-660) and ROX (533-610) channels.

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

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

Programming the Real-Time PCR Instrument

Interpretation of Patient Specimen Results

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

ORF1ab/S/M reaction mix:

SARS CoV-2 Targets			Internal Extraction Control	Result [†]
ORF1ab FAM (465-510)	S gene CY5 (618-660)	M gene ROX (575-610)	VIC/HEX/Yellow555 (533-580)	
Cq (+)	Cq (+)	Cq (+)	Cq (+) / (-)	SARS-CoV-2 Positive*
Cq (+)	Cq (+)	Cq (-)	Cq (+) / (-)	SARS-CoV-2 Positive*
Cq (+)	Cq (-)	Cq (+)	Cq (+) / (-)	SARS-CoV-2 Positive*
Cq (+)	Cq (-)	Cq (-)	Cq (+) / (-)	SARS-CoV-2 Positive*
Cq (-)	Cq (+)	Cq (-)	Cq (+) / (-)	SARS-CoV-2 Positive*
Cq (-)	Cq (-)	Cq (+)	Cq (+) / (-)	SARS-CoV-2 Positive*
Cq (-)	Cq (-)	Cq (-)	Cq (+)	SARS-CoV-2 Negative**
Cq (-)	Cq (-)	Cq (-)	Cq (-)	Result invalid, repeat testing of sample

FluA/FluB/RSV reaction mix:

Flu A FAM (465-510)	Flu B Cy5 (618-660)	RSV ROX (575-610)	Internal Extraction Control VIC/HEX/Yellow555 (533-580)	Result [†]
Cq (+)	Cq (-)	Cq (-)	Cq (+) / (-)	Flu A Positive*
Cq (-)	Cq (+)	Cq (-)	Cq (+) / (-)	Flu B Positive*
Cq (-)	Cq (-)	Cq (+)	Cq (+) / (-)	RSV Positive*
Cq (-)	Cq (-)	Cq (-)	Cq (+)	Test Negative**
Cq (-)	Cq (-)	Cq (-)	Cq (-)	Result invalid, repeat testing of sample

*All instances of FAM and/or Cy5 and/or ROX sample amplification in the ORF1ab/S/M testing indicates a SARS-CoV-2 positive sample. For the FluA/FluB/RSV test, all instances of FAM, Cy5, or ROX channel sample amplification indicates a positive result for the respective pathogen. Please manually inspect amplification curves for all samples assigned a Cq value to verify the positive amplification.

**If there is no target amplification in the FAM or Cy5 channels (plus ROX for the FluA/FluB/RSV test) for a test sample, before the result is confirmed as a true negative the Internal

Extraction VIC/HEX channel should be analysed. Positive amplification in the VIC/HEX channel confirms the PCR run is valid and the genesig® Winterplex RNA IEC added to the test sample during the RNA extraction process has been detected. The following acceptance criteria should be applied to confirm the negative samples:

- The IEC Cq value produced by the patient sample should be < 36 and should not exceed the NEC IEC Cq value + 6 (i.e. sample RNA IEC Cq < NEC RNA IEC Cq + 6). Failure to satisfy this criterion indicates a compromised sample extraction and an invalid result; testing of the sample must be repeated.

† Please note: Please check all channels even after identifying a positive result. The above interpretation table does not include the test case of more than one pathogen producing positive amplification, however this is a possibility. In the event of co-infection with more than one pathogen, positive amplification will be detected in the relevant channels.

Preventing Contamination

- Amplification technologies such as PCR are sensitive to accidental introduction of PCR product from previous amplifications 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 genesig® SARS-COV-2 Winterplex positive control template is provided in a sealed foil envelope and contains a mixture of high copy number 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. microcentrifuge 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.
- Always check the expiration date prior to use. Do not use expired reagent. Do not substitute or mix reagent from different kit lots or from other manufacturers.

- Change aerosol barrier pipette tips between all manual liquid transfers.
- During preparation of samples, compliance with good laboratory techniques is essential to minimize 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.
- When mixing reagents by pipetting up and down, this should be done with a volume roughly equal to 50% of the total component volume.
- DO NOT use water to resuspend the kit components. Use the appropriate buffers (provided with the kit) as instructed in the table in Section 2.
- Work surfaces, pipettes and centrifuges should be cleaned and decontaminated with cleaning products (e.g. 10% bleach, ethanol, DNA/RNA remover) to minimize risk of nucleic acid contamination.
- RNA samples should be maintained on a cold block or on ice during preparation and used to ensure stability.
- After each run has been set up and performed, clean work surfaces and equipment with a DNA/RNA remover.
- Handle post-amplification plates with care to ensure that the seal is not broken.
- Dispose of unused kit reagents and human specimens according to national regulations (refer to guidance detailed in Section 11).

Prevent DNase/RNase contamination

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

Specimen nucleic acid extraction kit/system

- Please consult the relevant Instruction For Use (IFU) and Safety Data Sheet (SDS), available from the manufacturer, before using your chosen extraction kit/ system.

9. Analytical performance

9.1. Analytical sensitivity (Limit of detection)

The limit of detection (LoD) is defined as the lowest concentration of analyte that could be reliably detected with 95% confidence. Briefly, negative anterior nasal swab samples stored in VTM, were contrived in the lysis stage of the extraction with wild type SARS-CoV-2 RNA provided by Twist Bioscience. The tentative LoD was tested at 3 contrivance levels: 20, 10 and 5 copies/reaction in the final PCR reaction. Each contrivance level was tested on 5 replicates for tentative LoD using the CFX Opus Real-Time PCR (Bio-Rad). The LoD of the SARS-CoV-2 assay was considered if at least one of the targets reached 95% confidence, i.e., ORF1ab, M and S genes, respectively. Similarly, for samples contrived pre-extraction with synthetic Flu A, Flu B and RSV RNA controls (Twist Bioscience), the tentative LoD was tested at 5 contrivance levels: 50, 20, 10, 5 and 3 copies/reaction in the final PCR reaction. Each contrivance level was tested in replicates of 5 and the tentative LoD was established at the lowest concentration where all 5 replicates give positive amplification on the CFX Opus Real-Time PCR System (Bio-Rad).

9.1.1 Verification of the LoD with anterior nasal swabs

Once the tentative LoD was established (100% positive call rate) for all targets, it was verified by testing the samples and contrivance in the same way as the tentative assay. Contrivance was diluted to required levels around the tentative LoD at each assay, giving a total of 20 replicates per target.

Results for the analytical sensitivity study using the CFX Opus Real-Time PCR instrument:

Verification of LoD data for SARS-CoV-2

Contrivance level in the PCR reaction (copies/rxn)	FAM (ORF1ab)		M gene (ROX)		S gene (Cy5)	
	Detect ion rate (%)	Mean Cq (STDEV)	Detect ion rate (%)	Mean Cq (STDEV)	Detect ion rate (%)	Mean Cq (STDEV)
30	100	32.12 (1.21)	100	33.26 (1.58)	100	31.48 (1.19)
20	100	32.64 (0.84)	100	33.75 (1.21)	100	32.14 (0.23)
10	100	33.39 (0.74)	85	34.39 (1.08)	100	33.07 (0.79)
5	95	34.30 (0.80)	85	34.16 (0.30)	100	33.53 (0.56)

Verification of LoD data for Flu A

Contrivance level (copies/rxn)	Detection rate (%)	FAM Mean Cq (STDEV)
3	100	36.27 (0.97)
2	90	37.16 (1.56)
1	65	37.18 (0.73)

Verification of LoD data for Flu B

Contrivance level (copies/rxn)	Detection rate (%)	Cy5 Mean Cq (STDEV)
5	100	32.11 (3.32)
3	80	32.65 (3.61)
2	95	33.23 (2.72)

Verification of LoD data for RSV

Contrivance level (copies/rxn)	Detection rate (%)	ROX Mean Cq (STDEV)
10	100	34.67 (0.44)
5	100	35.93 (0.81)
3	100	37.17 (0.88)

The data above demonstrates that the genesig® Real-Time PCR SARS-COV-2 Winterplex assay detects as little as 5 copies of SARS-Cov-2 whole viral genome per reaction ≥95% of the time for gene targets in any one of the three viral target detection channels. Similarly, the verification LoD where at least 95% of samples amplified, was determined to be 3 copies/reaction for Flu A, 2 copies/reaction for Flu B and 3 copies/reaction for RSV.

9.1.2 Analytical sensitivity with alternative sample types

The SARS-CoV-2 targets are the same as found in the genesig® COVID-19 3G assay, so for studies of other sample types, please see the IFU at <https://www.genesig.com/products/10061-genesig-covid-19-3g>.

To determine the sensitivity of the Flu A/Flu B/RSV assay tube with alternative sample types, saliva or oropharyngeal Virocult foam swabs were collected from donors negative for Flu A, Flu B, and RSV. Prior to extraction, samples were contrived with synthetic whole genome viral RNA from Twist Bioscience (Influenza A and Influenza B), or ATCC (RSV A and RSV B template) at the indicated contrivance levels. 20 replicates of each sample were assayed on the Cfx Opus Real-time PCR system, and are shown below:

Saliva samples contrived with Flu A

Contrivance level (copies/rxn)	Detection Rate	Mean HEX Cq (STDV)	Mean FAM Cq (STDV)
20	100%	22.69 (0.12)	36.85 (0.38)
15	100%	22.68 (0.17)	37.43 (0.46)
10	100%	22.97 (0.11)	38.06 (0.61)
6	90%	23.05 (0.1)	38.81 (3)

Saliva samples contrived with Flu B

Contrivance level (copies/rxn)	Detection Rate	Mean HEX Cq (STDV)	Mean FAM Cq (STDV)
20	95%	22.37 (0.09)	37.2 (0.94)
15	90%	22.51 (0.12)	37.51 (0.96)
10	60%	22.6 (0.1)	38.06 (0.73)

Saliva samples contrived with RSV

Contrivance level (copies/rxn)	Detection Rate	Mean HEX Cq (STDV)	Mean FAM Cq (STDV)
20	100%	23.03 (0.1)	36.57 (0.7)
15	80%	23.47 (0.09)	37.13 (0.81)
10	95%	23.03 (0.08)	37.87 (0.73)
6	85%	22.78 (0.17)	38.17 (0.57)

Oropharyngeal swabs contrived with Flu A

Contrivance level (copies/rxn)	Detection Rate	Mean HEX Cq (STDV)	Mean FAM Cq (STDV)
3	95%	24.10 (0.09)	39.33 (1.02)
2	95%	23.98 (0.06)	39.33 (0.90)
1	65%	24.37 (0.12)	40.63 (1.28)

Oropharyngeal swabs contrived with Flu B

Contrivance level (copies/rxn)	Detection Rate	Mean HEX Cq (STDV)	Mean Cy5 Cq (STDV)
5	100%	25.07 (0.07)	36.42 (0.77)
3	95%	23.28 (0.08)	37.17 (0.80)
1	35%	24.23 (0.08)	38.82 (1.48)

Oropharyngeal swabs contrived with for RSV

Contrivance level (copies/rxn)	Detection Rate	Mean HEX Cq (STDV)	Mean ROX Cq (STDV)
10	100%	23.24 (0.07)	35.80 (0.61)
5	95%	23.60 (0.08)	36.88 (0.58)
3	90%	24.00 (0.14)	37.76 (0.55)

In summary the confirmed LoDs for each pathogen are as follows:

Confirmed LoDs for Both Sample Types for Each Pathogen (copies per reaction)

Sample type	Flu A	Flu B	RSV
Saliva	10	20	10
Oropharyngeal	2	3	5

Investigation of assay sensitivity using nasopharyngeal (NP) swabs has not been performed experimentally. However, a study by Monita et al. (2021, Clinical Infectious Dis 72: 482-485, <https://doi.org/10.1093/cid/ciaa759>) showed that, among 146 NP and oropharyngeal (OP) paired swabs collected and subjected to subsequent real-time reverse transcriptase polymerase chain reaction (RT-qPCR) testing, NP swab cycle threshold values were lower (indicating more virus) in 66.7% of concordant-positive pairs, suggesting NP swabs may detect SARS-CoV-2 more accurately. Further published studies also suggested the same results, with NP swabs yielding higher SARS-CoV-2 RNA concentrations compared to OP swabs. (Yang et al (2020) Infection. Innovation (N Y). 2020 Nov 25;1(3):100061. doi: 10.1016/j.xinn.2020.100061; Hitzenbichler et al (2021) Viruses. 2021;13:653. doi:10.3390/v13040653; and Wang et al (2020) nt J Infect Dis. 2020 May; 94:107-109. doi: 10.1016/j.ijid.2020.04.023.)

The evidence provided by published literature utilising true clinical samples demonstrates that NP swabs generate better sensitivity and accuracy compared to OP swabs. The LoD of an assay is reported as the LoD of the sample matrix that is shown to provide the least sensitivity. This provides assurance of detection and positive identification of the stated sample matrices at assay LoD. As NP swab matrix has been demonstrated to produce greater sensitivity than OP swab matrix, the LoD for both these sample types has been experimentally determined using OP swab matrix for this assay.

9.1.3 Analytical sensitivity with alternative instruments

The SARS-CoV-2 targets are the same as found in the genesig® COVID-19 3G assay, so for studies of other instruments, please see the IFU at <https://www.genesig.com/products/10061-genesig-covid-19-3g>. The previous version of the Flu A/Flu B/RSV tube of the Winterplex assay was validated on the Lightcycler 480 II and the Applied Biosystems 7500 instruments, and data on validation of the revised version of the kit will be included in a future iteration of this IFU. At launch, we have validated the revised assay for use on the Bio-Rad Cfx Opus (see section 9.1.1) and the genesig q32 Real-time PCR instrument. As before, anterior nasal swabs from negative donors were contrived with synthetic whole genome viral RNA from Twist Bioscience (Influenza A and Influenza B), or ATCC (RSV A and RSV B template) at the indicated contrivance levels. 20 replicates of each sample were assayed on the q32 instrument, with results as shown below:

Verification of LoD Data for Influenza A using the q32 instrument

Concentration (copies/rxn)	Total Replicates	Detection Rate (%)	Mean HEX Cq (STDV)	Mean FAM Cq (STDV)
3	20	18 (90)	20.17 (0.21)	35.92 (0.89)
2	20	18 (90)	20.13 (0.31)	36.54 (1.19)
1	20	15 (75)	20.06 (0.24)	37.06 (1.48)

Table 12. Verification of LoD Data for Influenza B using the q32 instrument

Concentration (copies/rxn)	Total Replicates	Detection Rate (%)	Mean HEX Cq (STDV)	Mean Cy5 Cq (STDV)
5	20	20 (100)	21.09 (0.17)	34.66 (0.55)
3	20	8 (40)	23.07(1.73)	36.53 (0.89)
1	20	20 (100)	20.69 (0.18)	35.69 (0.68)

Table 13. Verification of LoD Data for RSV-A and RSV-B using the q32 instrument

Concentration (copies/rxn)	Total Replicates	Detection Rate (%)	Mean HEX Cq (STDV)	Mean ROX Cq (STDV)
10	19	19 (100)	21.37 (0.06)	34.73 (0.51)
5	20	20 (100)	20.06 (0.10)	35.41 (0.72)
3	20	20 (100)	20.78 (0.08)	36.22 (0.86)

For Influenza A, although no contrivance levels tested produced $\geq 95\%$ positive amplification, the lowest contrivance level tested where $\geq 90\%$ of samples amplified was 2 copies/reaction. Therefore, the LoD₉₀ using the genesig® q32 is 2 copies/reaction for synthetic Influenza A template.

For Influenza B, the lowest contrivance level below which $>95\%$ of samples showed positive amplification was 5 copies/reaction. This matches the verification LoD reported in the Winterplex Analytical Sensitivity QF36 (carried out using the CFX Opus Real-Time PCR System). Therefore, the LoD using the genesig® q32 is 5 copies/reaction for synthetic Influenza B RNA template.

Finally, for RSV-A and RSV-B, the lowest contrivance level tested where $\geq 95\%$ of samples showed positive amplification was 3 copies/reaction. This matches the verification LoD reported in the Winterplex Analytical Sensitivity QF36 (carried out using the CFX Opus Real-Time PCR System). Therefore, the LoD using the genesig® q32 is 3 copies/reaction for synthetic RSV-A and RSV-B template

9.2. Analytical specificity (Cross reactivity)

The objective of this study is to assess the Analytical Specificity, i.e., inclusivity and exclusivity for the genesig® Real-Time PCR SARS-COV-2 Winterplex 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 genesig® Real-Time PCR SARS-COV-2 Winterplex assay. The *in silico* analysis also evaluated assay inclusivity.

9.2.1 Latest *in silico* Specificity Analysis

To ensure the COVID-19 primer/probe remain specific to detect SARS-CoV-2 genomes, Primerdesign's bioinformaticians review daily the SARS-CoV-2 sequence submissions on the GISAID EpiCoV database. As of 16th of September 2021, *in silico* analysis confirms the COVID-19 assay primers and probe still show 99.9%, 99.5% and 99.9% detection with the 3,106,281, 3,089,798 and 2,987,772 full length, good quality SARS-CoV-2 sequences at ORF1ab, S and M genes respectively, as published on the GISAID EpiCoV database. The GISAID database was used to determine that the primers and probe sets will detect over 95% of sequences for Flu A (35,900 sequences analysed in EpiFlu on 22nd June 2021), Flu B (26,724 sequences analysed in EpiFlu on 6th August 2021) and RSV (2,678 sequences analysed from EpiRSV on 23rd June 2021)

9.2.2 Wet testing

Related pathogens and pathogens that are likely to be present in the clinical sample have been evaluated *in silico* to identify the homology between the primers/probe of the assay and the pathogens. This is confirmed with testing of the assays against other viral targets that could be present in a clinical sample.

***In vitro* testing:**

For SARS-CoV-2 testing, 5 panels were sourced:

NATrol™ Pneumonia panel (ZeptoMetrix)

NATrol™ Coronavirus-SARS Stock (ZeptoMetrix)

Respiratory Evaluation Panel (Qnostics, Scotland, UK)

QCMD from the 2019 Coronavirus EQA programme (Qnostics)

QCMD from the 2019 MERS Coronavirus EQA Programme (Qnostics)

The samples from these panels are representative of true clinical samples and evaluated by the SARS-CoV-2 primers and probes of the genesig® Real-Time PCR SARS-COV-2 Winterplex assay. The results of the *in vitro* cross-reactivity testing are presented below:

		genesig® Real-Time PCR SARS-COV-2 Winterplex assay – SARS-CoV-2 tube			
Sample number	Panel member	FAM (ORF1a b)	HEX/VI C (IEC)	ROX (M gene)	Cy5 (S gene)
1	<i>A.baumannii</i>	N/A	25.5	N/A	N/A
2	<i>E.cloacae</i>	N/A	21.5	N/A	N/A
3	<i>E.coli</i>	N/A	22.4	N/A	N/A
4	<i>H.influenzae</i>	N/A	24.5	N/A	N/A
5	<i>K.oxytoca</i>	N/A	22.7	N/A	N/A
6	<i>P.aeruginosa</i>	N/A	23.3	N/A	N/A
7	<i>P.mirabilis</i>	N/A	23.9	N/A	N/A
8	<i>S.agalactiae</i>	N/A	23.8	N/A	N/A
9	<i>S.marcescens</i>	N/A	19.6	N/A	N/A
10	<i>S.pneumoniae</i>	N/A	24.0	N/A	N/A
11	<i>S.pyogenes</i>	N/A	24.9	N/A	N/A
12	<i>K.pneumoniae</i>	N/A	24.3	N/A	N/A
13	<i>K.pneumoniae</i>	N/A	23.7	N/A	N/A
14	<i>K.pneumoniae</i>	N/A	23.6	N/A	N/A
15	Coronavirus-SARS	N/A	24.5	N/A	N/A
16	INF A H1N1	N/A	23.9	N/A	N/A
17	INF A H3N2	N/A	24.4	N/A	N/A
18	INF B Victoria	N/A	23.9	N/A	N/A
19	INF B Yamagata	N/A	24.1	N/A	N/A
20	RSV A	N/A	24.1	N/A	N/A
21	RSV B	N/A	23.4	N/A	N/A
22	Coronavirus-NL63	N/A	24.1	N/A	N/A
23	Coronavirus-229E	N/A	23.8	N/A	N/A
24	Coronavirus-HKU	N/A	24.6	N/A	N/A
25	Coronavirus-OC48	N/A	24.0	N/A	N/A
26	MERS Coronavirus	N/A	23.9	N/A	N/A
Positive extraction control	Extracted SARS-CoV-2 Medium Q Control (mean)	31.2	24.8	29.6	28.2

PCT	genesig® COVID-19 3G Positive control	17.2	NA	15.2	14.7
NTC	NTC	N/A	N/A	N/A	N/A

To determine the specificity of the FluA/FluB/RSV tube the NATrol™ Pneumonia Verification Panel (NATPPQ-BIO) from ZeptoMetrix, NATrol™ Influenza/RSV Verification panel (NATFRVP-C) from ZeptoMetrix, and the Qnostics Respiratory Target Multiplex controls panel were used. Since some overlap of organisms exists within these panels, any duplicates were removed. Some of the panel members pool more than one target within the sample, so in those cases all panel members are listed, with intended targets of the genesig® Real-Time PCR SARS-COV-2 Winterplex assay shown in bold type.

Sample identity	Panel member	Cq obtained			
		FAM (Flu A)	ROX (RSV)	Cy5 (Flu B)	HEX (IEC)
PTC Average Cq	n/a	19.91	19.81	15.23	n/a
NTC Average Cq	n/a	n/a	n/a	18.73*	25.02
NEC Average Cq	n/a	n/a	n/a	31.85*	n/a
1	Adenovirus Type 3	n/a	n/a	n/a	28.19
2	Adenovirus Type 31	n/a	n/a	n/a	27.24
3	<i>C. pneumoniae</i> CWL-029	n/a	n/a	n/a	27.50
4	Influenza A H1N1 (A/NY/02/09)*	32.39	n/a	n/a	27.08
5	Influenza A H3 (A/Brisbane/10/07)*	35.38	n/a	n/a	27.20
6	Metapneumovirus 8 Peru6-2003	n/a	n/a	n/a	25.41
7	<i>Mycoplasma pneumoniae</i> (M129)	n/a	n/a	n/a	27.39
8	Parainfluenza Type 1	n/a	n/a	n/a	27.06
9	Rhinovirus 1A	n/a	n/a	n/a	25.98
10	Coronavirus NL63	n/a	n/a	n/a	26.99
11	Influenza A H1 A/New Caledonia/20/99	31.68	n/a	n/a	27.52
12	Influenza B/Florida/02/06	n/a	n/a	26.02	27.35

13	Respiratory Syncytial Virus A	n/a	26.71	n/a	27.00
14	Respiratory Syncytial Virus B (CH93(18)-18)	n/a	24.31	n/a	26.61
15	<i>Legionella pneumophila</i> (Philadelphia)	n/a	n/a	n/a	27.11
16	Influenza A Type H1N1	36.40	35.04	40.15	26.12
	Influenza B Type Victoria				
	Respiratory Syncytial Virus Type A				
	SARS-CoV-2				
17	Parainfluenza Type 1	n/a	n/a	n/a	26.13
	Adenovirus Type 1				
	<i>Mycoplasma pneumoniae</i>				
	Coronavirus Type OC43				
18	Parainfluenza Type 2	n/a	n/a	n/a	26.68
	Metapneumovirus Type A2				
	Enterovirus Type A16				
	Coronavirus Type 229E				
19	Parainfluenza Type 3	n/a	n/a	n/a	26.44
	Rhinovirus Type 16				
	<i>Legionella pneumophila</i>				
	Coronavirus Type NL63				
20	Parainfluenza Type 4	n/a	30.48	n/a	26.80
	Enterovirus Type 68				
	Adenovirus Type 14				
	Respiratory Syncytial Virus Type B				
21	4+12+13	35.78	28.22	27.55	27.06

*While some NTC and NEC samples gave a Cq value, visual analysis of the run showed no amplification.

Overall, this data confirms that the genesig® Real-Time PCR SARS-COV-2 Winterplex assay maintains the expected inclusivity and exclusivity for the intended targets of SARS-CoV-2, Flu A, Flu B, and RSV.

9.3. Precision (repeatability and reproducibility)

The objective of this study is to determine the precision of three different batches of genesig® Real-Time PCR SARS-CoV-2 Winterplex assay.

To assess the precision of this assay, four variables are normally analysed: Inter-site, inter-day, inter-instrument and inter-operator variability. Data for inter-operator, inter-instrument and inter-day variability of the genesig® COVID-19 3G Real-Time PCR assay (see COVID-19 3 gene IFU at <https://www.genesig.com/products/10061-genesig-covid-19-3g>), and Influenza A, RSV-A and RSV-B has been previously validated (see Winterplex 2020 IFU at https://www.genesig.com/assets/files/Path_SARS_CoV_2_Winterplex_IFU_Issue_102.pdf for associated data). As such, this study is validating:

- The inter-instrument, inter-operator and inter-day variability for Influenza B.
- The inter-site variability of Influenza A, Influenza B, RSV-A and RSV-B, and the SARS-CoV-2 Orf1ab, M gene and S gene region

Anterior nasal swabs were collected from donors who were negative for SARS-CoV-2, Influenza A, Influenza B and RSV-A and B. The swabs were collected using Virocult foam swabs and placed in Viral Transport Medium (VTM). Swab extractions were carried out using the KingFisher™ Flex Purification System in conjunction with the exsig™ Mag Extraction System. Prior to extraction, samples were contrived with synthetic whole genome viral RNA from Twist Bioscience (H1N1 Influenza A, Influenza B, and RSVA and RSV B) or ATCC (SARS-CoV-2 whole genome). Three contrivance levels of each whole viral genome was assessed, using three different batches of the genesig® Real-Time PCR SARS-CoV-2 Winterplex assay. Cfx Opus Real-time PCR systems were used as indicated below, and the statistic used to determine the variation between sites, operators, instruments and days, and hence the precision of the kit, was the coefficient of variance (CV). In all cases this was calculated from the mean and standard deviation of the Cq obtained by 20 replicates at each contrivance level. A CV lower than 10% was considered desirable and a CV lower than 15% was considered acceptable

Inter-site precision - coefficient of variance (%) for all targets in all batches

Batch	Channel (target)	Contrivance level (x LoD)		
		15x	10x	5x
Batch 1	FAM (ORF1ab)	1.99	1.49	1.54
	HEX (IEC)	2.39	2.05	2.49
	ROX (M gene)	2.09	1.52	2.79
	Cy5 (S gene)	1.82	1.06	0.69
	FAM (Flu A)	2.07	1.06	3.95
	ROX (RSV)	1.28	1.80	1.35
	Cy5 (Flu B)	2.61	2.57	2.21
Batch 2	FAM (ORF1ab)	1.56	1.12	1.52
	HEX (IEC)	0.89	2.17	1.58
	ROX (M gene)	3.13	1.58	2.54
	Cy5 (S gene)	1.78	1.19	1.95
	FAM (Flu A)	1.42	2.16	1.98
	ROX (RSV)	2.49	1.76	1.64
	Cy5 (Flu B)	3.85	3.81	3.41
Batch 3	FAM (ORF1ab)	2.20	1.13	1.85
	HEX (IEC)	1.94	0.83	4.18
	ROX (M gene)	3.50	1.38	2.24
	Cy5 (S gene)	3.48	1.28	0.98
	FAM (Flu A)	2.15	1.15	1.81
	ROX (RSV)	1.24	1.81	1.10
	Cy5 (Flu B)	1.09	1.17	1.10

Coefficient of variance (%) for Flu B between operators, Instruments and days of assay

Batch	Channel (target)	Inter operator			Inter instrument			Inter day		
		Contrivance level (x LoD)			Contrivance level (x LoD)			Contrivance level (x LoD)		
		15x	10x	5x	15x	10x	5x	15x	10x	5x
Batch 1	HEX (IEC)	1.39	1.79	0.87	0.74	2.17	2.39	1.15	1.36	4.40
	Cy5 (Flu B)	1.10	1.18	1.28	0.86	0.75	1.19	1.19	0.84	0.86
Batch 2	HEX (IEC)	0.90	2.00	1.22	0.95	1.20	0.64	1.91	1.37	1.78
	Cy5 (Flu B)	2.93	3.86	3.27	0.48	0.62	0.69	2.39	2.62	3.43
Batch 3	HEX (IEC)	2.18	2.59	1.90	1.28	1.03	1.12	1.52	1.61	4.61
	Cy5 (Flu B)	1.59	1.15	2.23	0.75	0.74	0.87	1.04	1.61	1.79

These data show that the Winterplex kit produces precise results across the channels of interest. The coefficient of variance produced between sites, operators, instruments and days is of a desirable level as all coefficients of variance are below 10%. Coupled with the results of the other studies on the precision of the genesig® COVID-19 3G Real-Time PCR assay, and the previous version of the Winterplex assay, we conclude that the assay performs consistently.

9.4. Accuracy (trueness and precision)

The objective of this study was to determine the diagnostic accuracy of the genesig® Real-Time PCR SARS-CoV-2 Winterplex assay through the generation of three key statistics.

- Positive Percentage Agreement (PPA)
- Negative Percentage Agreement (NPA)
- Overall Percentage Agreement (OPA)

Percentage agreement was generated by conducting single-blind randomized testing using the Winterplex assay and comparing the results to the contrivance status of 60 total samples for the SARS-CoV-2 targets (30 contrived with Twist synthetic wild type SARS-CoV-2, 30 negative samples), and 120 total samples for the Flu A/Flu B/RSV tube:

- 30 contrived with Twist synthetic Flu A at 5 x LoD (15 copies/reaction)

- 30 contrived with Twist synthetic Flu B at 5 x LoD (25 copies/reaction)
- 30 contrived with RSV ATCC at 5 x LoD (15 copies/reaction)
- 30 samples negative for all viruses

		Randomized contrived samples		
		Positive	Negative	Total
Samples contrived with Flu A RNA	Positive	30	0	30
	Negative	0	30	30
	Total	30	30	60

Agreement	Level
OPA	100%
PPA	100%
NPA	100%

Samples were tested blind with genesig® Real-Time PCR SARS-COV-2 Winterplex assay and compared with the contrivance status to produce the percentage agreements. Briefly, 60 negative for SARS-CoV-2 saliva samples were collected from 5 donors and extracted with the KingFisher™ Flex Purification System in conjunction with exsig™ Mag Extraction System. 30 samples were contrived at 5x the LoD, as defined in Analytical Sensitivity. Samples were contrived with synthetic SARS-CoV-2 RNA provided by Twist BioScience. The remaining 30 samples were not contrived and remained negative. The below tables show the result summary for SARS-CoV-2:

		Randomized contrived samples		
		Positive	Negative	Total
Samples contrived with Flu B RNA	Positive	30	0	30
	Negative	0	30	30
	Total	30	30	60

genesig® COVID-19 3G: Results for the blind contrivance accuracy study using genesig® COVID-19 3G.

		Randomized contrived samples		
		Positive	Negative	Total
Samples contrived with wild type SARS-COV-2 RNA	Positive	30	0	30
	Negative	0	30	30
	Total	30	30	60

Agreement	Level
OPA	100%
PPA	100%
NPA	100%

Agreement	Level
OPA	100%
PPA	100%
NPA	100%

		Randomized contrived samples		
		Positive	Negative	Total
Samples contrived with RSV RNA	Positive	30	0	30
	Negative	0	30	30
	Total	30	30	60

Agreement	Level
OPA	100%
PPA	100%
NPA	100%

Separately, 90 Flu A/Flu B/RSV negative swabs in VTM were collected, 30 of these were each contrived with Flu A synthetic whole genome RNA from (Twist Bioscience), Flu B (Twist) or RSV (ATCC) at 5x LoD. A further 30 negative VTM swabs were processed. All 120 blinded assays were performed with the Flu A/Flu B/RSV tube, with samples yielding amplification in the FAM channel deemed as Flu A positive, the Cy5 channel as Flu B positive, and the ROX channel as RSV positive.

All Percentage Agreements generated in this study were above the required specification of >90%. The reported Overall Percentage Agreement for the genesig® SARS-CoV-2 Winterplex assay when detecting SARS-CoV-2. Flu A, Flu B and RSV was 100%.

9.5. Interfering substances

This revised genesig® SARS-CoV-2 Winterplex assay shows a level of precision that is below 10% CV on all comparisons (inter-site, inter-operator, inter-instrument, and inter-day – section 9.3), and the accuracy has an OPA >95% for all targets. Therefore the study on Interfering Substances available in the Winterplex 2020 IFU ([https://www.genesig.com/assets/files/Path SARS CoV 2 Winterplex IFU Issue 102.pdf](https://www.genesig.com/assets/files/Path_SARS_CoV_2_Winterplex_IFU_Issue_102.pdf)) and the genesig® COVID-19 3G assay (IFU at <https://www.genesig.com/products/10061-genesig-covid-19-3g>) can be used as equivalent studies. In summary, the following twelve interfering substances were tested for their effect on the Cq of each target:

Interfering Substance
Blood (Haemoglobin)
Nasacort Allergy Nasal Spray (Triamcinolone acetonide)
Dymista Allergy Nasal Spray (Corticosteroids – Azelastine hydrochloride & Fluticasone)
Corticosteroid – Dexamethasone
*Corticosteroids – Fluticasone
Antiviral medication – Guaifenesin
Antiviral medication – Oseltamivir
Antibacterial medication – Mupirocin
Antibacterial eye drops – Tobramycin
Throat lozenge (Strepsils – 2,4-Dichlorobenzyl Alcohol, & Amylmetacresol)
Mucin
α-Amylase

None of the 12 substances interfered with the SARS-CoV-2 targets in the genesig® COVID-19 3G assay. 3 were found to have a significant effect on the SARS-CoV-2 Winterplex assay – Nasacort, Dymista and Mucin.

Nasacort was shown to impact the number of replicates amplified past 10% v/v for all targets (Flu A, Flu B and RSV). Dymista had a similar effect (impacting the number of replicates amplifying past 10% v/v), however only for the Flu A target. Finally, although Mucin was shown to have a significant effect on COVID S, Flu A and Flu B during the screen, the dose response suggested this drop in assay

performance does not worsen with increased Mucin concentration

10. Clinical Performance

Clinical performance validation of the SARS-CoV-2 target tube used an existing evaluation of the *in vitro* diagnostic performance of the genesig® COVID-19 3G assay, which has the same formulation as the SARS-CoV-2 target tube of the genesig® SARS-CoV-2 Winterplex assay. Samples were previously collected from patients suspected of having COVID-19 at the Queen Elizabeth Hospital, NHS Gateshead, both symptomatic and asymptomatic.

The contingency table below illustrates the total positives and negatives that were used to calculate the Diagnostic Sensitivity (PPA), Diagnostic Specificity (NPA), Positive Predictive Value, (PPV) and Negative Predictive Value (NPV) and the 95% confidence interval (CI) for sensitivity and specificity. Of the 471 samples, 157 gave true positive results and 314 gave true negative results. This resulted in diagnostic sensitivity of 100% (95% CI 97.7%-100%) and diagnostic specificity of 100% (95% CI 98.9%-100%).

Contingency table for genesig® COVID-19 3G Clinical Performance Evaluation

		Comparator assay (TaqPath) / Resolver assay (genesig® COVID-19)		
		Positive	Negative	Total
genesig® COVID-19 3G	Positive	157	0	157
	Negative	0	314	314
	Total	157	314	471

Clinical performance of genesig® COVID-19 3G

	%	95% CI
Diagnostic Sensitivity	100%	97.7% - 100%
Diagnostic Specificity	100%	98.9% - 100%
PPV	100%	-
NPV	100%	-

Clinical performance of the Flu A/Flu B/RSV tube, was evaluated with the previous genesig® SARS-CoV-2 Winterplex assay, which has a different primer/probe combination for Flu B. To first confirm that the previous and

new versions perform equivalently, the table below compares the accuracy of the assays against contrived samples.

Comparison of PPA, NPA and OPA (%) from accuracy experiments between the previous Winterplex assay (2020) and new Winterplex assay (2021)

	Target							
	COVID-19*		Flu A		Flu B		RSV	
	2020	2021	2020	2021	2020	2021	2020	2021
PPA	100%	100%	96%	100%	100%	100%	100%	100%
NPA	100%	100%	100%	100%	100%	100%	100%	100%
OPA	100%	100%	98%	100%	100%	100%	100%	100%

*Tube 1 Winterplex assay had previously (2020) consisted of the genesig® COVID-19 2G whilst the updated (2021) version has replaced it with genesig® COVID-19 3G.

As this shows, that previous and updated Winterplex have equivalent performance, data from the Performance Evaluation study at Tallaght University Hospital (Dublin, Ireland) can be considered here. A total of 132 results were obtained and the Flu A/Flu B/RSV tube of the Winterplex assay was shown to perform in line with the comparator assay (TibMol Biol multiplex). There was a single false positive or negative with each target, and the diagnostic sensitivity and specificity obtained can be seen in the table below.

		Comparator assay (TiBMolBiol)		
		Positive	Negative	Total
Influenza A	Positive	38	0	38
	Negative	1	93	93
	Total	39	93	132

		Comparator assay (TiBMolBiol)		
		Positive	Negative	Total
Influenza B	Positive	29	1	30
	Negative	0	102	103
	Total	29	103	132

		Comparator assay (TiBMolBiol)		
		Positive	Negative	Total
RSV	Positive	16	1	17

	Negative	0	114	114
	Total	16	115	131

Diagnostic Sensitivity and Specificity of the previous Winterplex Flu A/Flu B/ RSV

	Diagnostic Sensitivity (%)	Diagnostic Specificity (%)
Flu A	97.4	100
Flu B	100	99
RSV	100	99.1

Overall, these results show that the genesig® SARS-CoV-2 Winterplex assay detects each target with a diagnostic sensitivity and diagnostic specificity >95%, and that the previous and updated Winterplex assays have equivalent clinical performance. A prospective, multi-centre clinical performance study which will take place in NHS sites will start on 28th September 2021.

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

- Brand name: PrimerdesignTM Ltd
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13. Technical Support

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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