Instructions for Use (IFU)

genesig® Real-Time PCR SARS-CoV-2 Winterplex CE IVD kit
96 tests

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1. Intended purpose

The genesig® Real-Time PCR SARS-CoV-2 Winterplex CE IVD kit 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 combined oropharyngeal/anterior nasal swab specimens. This multiplex assay provides rapid screening of individuals suspected of SARS-CoV-2, Flu A, Flu B and RSV infections and aids in 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 CE IVD kit 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 CE IVD kit. This time includes the time to extract nucleic acid from a specimen, PCR set-up, PCR run time, and availability of results.

2. Summary

The genesig® Real-Time PCR SARS-CoV-2 Winterplex CE IVD kit 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 combined oropharyngeal/anterior nasal swab 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 a 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 CE IVD kit.

The genesig® Real-Time PCR SARS-CoV-2 Winterplex CE IVD kit 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® Real-Time PCR SARS-CoV-2 Winterplex CE IVD kit 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 targets specific probes for the detection of the amplified RNA. The probes are labelled with fluorescent reporter and quencher dyes.

RNA isolated and purified from combined oropharyngeal/anterior nasal swab specimens is reverse transcribed to cDNA and subsequently amplified using a validated Real-Time PCR instrument: genesig® q32 Real-Time PCR instrument, Bio-Rad CFX Opus Real-time PCR Detection System (Maestro software 2.0, version 5.0), Roche LightCycler 480 II and Qiagen Rotor-Gene Q thermal cycler. 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 CE IVD kit 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 a sealed foil pouch	Master mix resuspension buffer
ORF1ab/S/M-CE Primer/probe Mix including IEC	1	220*	Amber, vial stored in a sealed foil pouch	Template preparation buffer
FluA/FluB/RSV-CE Primer/probe Mix including IEC	1	220*	Yellow (Amber body), vial stored in a 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-CE Positive control template	1	800*	Red, vial stored in a sealed foil pouch	Template preparation buffer
genesig® Winterplex IEC RNA CE	2	1000*	Blue, vial stored in a sealed foil pouch	

*The projected volume once resuspended

The genesig® Real-Time PCR SARS-CoV-2 Winterplex CE IVD kit Primer & Probe Mix consists of two tubes. The 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 labelled 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 Internal extraction control (IEC) RNA. The FluA/FluB/RSV primer & probe tube contains the primers and FAM-labelled probe for the detection of Influenza A, primers and Cy5 labelled probe specific to Influenza B, primers and ROX labelled probe for RSV, and the primers and HEX labelled probe for the genesig® Winterplex IEC RNA.

The OneStep Lyophilised Master Mix, the two Primers & Probes mixes, genesig® Real-Time PCR SARS-CoV-2 Winterplex Positive control template and genesig® Winterplex IEC RNA 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 CE IVD kit is shipped at ambient temperatures but must be stored at -20°C upon arrival.
- The genesig® Real-Time PCR SARS-CoV-2 Winterplex CE IVD kit should be stored in the original packaging and is stable for up 18 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 before 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 CE IVD kit 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 genesig® Real-Time PCR SARS-CoV-2 Winterplex CE IVD kit 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 28 January 2021: Laboratory biosafety guidance related to coronavirus disease (COVID-19): https://www.who.int/publications/i/item/WHO-WPE-GIH-2021.1
- Refer to Interim Guidelines for Collecting and Handling of Clinical Specimens for COVID-19 Testing: https://www.cdc.gov/coronavirus/2019-ncov/lab/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 -70ºC or lower.
- Refer to Section "Collecting the Specimen" for guidance.

8. Assay procedure

Reagent and Controls Preparation

OneStep Lyophilised Master Mix preparation

- Upon receipt, the dried master mix can be stored at -20ºC. Do not use it 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 -20ºC. The resuspended master mix is stable for one month when stored at -20ºC.
- 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 -20ºC.

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 probes for the detection of the IEC.
- Upon receipt, the dried primers/probes mix can be stored at -20ºC. Do not use it after the expiry date (see product label).
- Precautions: this reagent should only be handled in a clean area and not exposed to light.
- Using the aseptic technique, resuspend each dried primer/probe mix in 220 µl of Template preparation buffer and vortex to mix.
- Store at -20ºC. The resuspended primer/probe mix is stable for one month when stored at -20ºC.
- 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 -20ºC.
- 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-CE 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 -20ºC. Do not use it after the expiry date (see product label).
- Using the aseptic technique, resuspend the dried PCT in 800 µl of Template preparation buffer and vortex thoroughly. Resuspended PCT is stable for one month when stored at -20ºC.
- 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 -20ºC.
- 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 in the ROX channel for the M gene (SARS-CoV-2) and RSV.

genesig® Winterplex RNA Internal extraction control (IEC) preparation

The genesig® Winterplex Internal extraction control (IEC) RNA 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 -20ºC. Do not use it after the expiry date (see product label).
- Using the aseptic technique, resuspend the dried IEC in 1000 µl of Template preparation buffer and, and vortex thoroughly. Resuspended IEC is stable for one month when stored at -20ºC.
- 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 -20ºC.

Negative Extraction Control (NEC) preparation

- Prepare at least one negative extraction control (NEC) every time RNA is extracted from a cohort of samples. In addition, each PCR run should include a minimum of one NEC, prepared in parallel with the clinical samples.
- The NEC is extracted 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 test 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 (see the section on NEC preparation above).
- 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 before 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 CE IVD kit 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 the 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

	Combined oropharyngeal/anterior nasal swabs
Collection	Swabs: Dacron or polyester flocked swabs in viral transport medium
Transport temperature*	2-8ºC ≤ 72 hrs
Short-term storage (pre-extraction) *	2-8ºC ≤ 72 hrs
Long-term storage (pre-extraction) *	≤ -70ºC for longer periods
Extraction sample volume	550 µL**
Extraction elution volume	50 µL

*These are CDC recommendations: Interim Guidelines for Collecting and Handling of Clinical Specimens for COVID-19 Testing: https://www.cdc.gov/coronavirus/2019-ncov/lab/guidelines-clinical-specimens.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 genesig® Real-Time PCR SARS-CoV-2 Winterplex CE IVD kit are 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 is processed for RNA extraction (i.e. an extraction with no clinical specimen/sample added).
- The genesig® Winterplex Internal extraction control (IEC) RNA 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 that 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, PCT, 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.

a) Resuspend each of the two primer/probe tubes in 220 µl of template preparation buffer, vortex to mix.
b) Resuspend the OneStep Lyophilised Master Mix in 525 µl Master Mix Resuspension Buffer, gently swirl to mix.
c) Plate set-up configuration can vary with the number of specimens. An NEC must be included in each plate set-up (refer to Sections 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)
    1. A The PCT will be added after all other reagents and samples have been added to the plate.
    2. This will be in an area for handling nucleic acid and away from the NEC, NTC and any clinical specimen/ samples.
    3. This is to prevent plate set-up, reagent, or specimen contamination with the PCT.
d) 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 an excess reaction mix to allow for pipetting error. Use the following guide to determine the volume of reagents to add to the reaction mix:
    1. If the number of samples (n) is ≤ 10, then N = n+1
    2. If number of samples (n) is > 10 and ≤ 20, then N = n+2
    3. If number of samples (n) is > 20, then N = n+ 10% of total number of samples
e) 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/M or FluA/FluB/RSV)	2*

*Multiply all numbers by (N). Refer to step (d) above, to ensure there is a 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:
    1. NEC (please refer to Section 8 – Negative Extraction Control (NEC) Preparation)
    2. NTC (please refer to Section 8 – No Template Control)

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 it in the instrument.

Programming the Real-Time PCR 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))
- LightCycler 480 instrument Operator's manual (July 2016, Addendum 4, Software version 1.5)
- Rotor-Gene Q thermal cycler (Q-Rex Software v1.0)
- genesig® q32 user manual (2022)

For the genesig® q32 instrument (Primerdesign, Novacyt Group, software version 1.5 or greater), please use the genesig® Multiplex module and program the instrument as described in the user manual.

The tables below contain the information required for inputting in the "Targets and Tests" section of the Setup tab:

Test/Tube 1

Use	Dye	Color	Name
Yes	FAM	User Preference	ORF1ab
Yes	HEX	User Preference	IEC
No	ROX	User Preference	M gene
Yes	Cy5	User Preference	S gene

Test/Tube 2

Use	Dye	Color	Name
Yes	FAM	User Preference	Flu A
Yes	HEX	User Preference	IEC
Yes	ROX	User Preference	RSV
Yes	Cy5	User Preference	Flu B

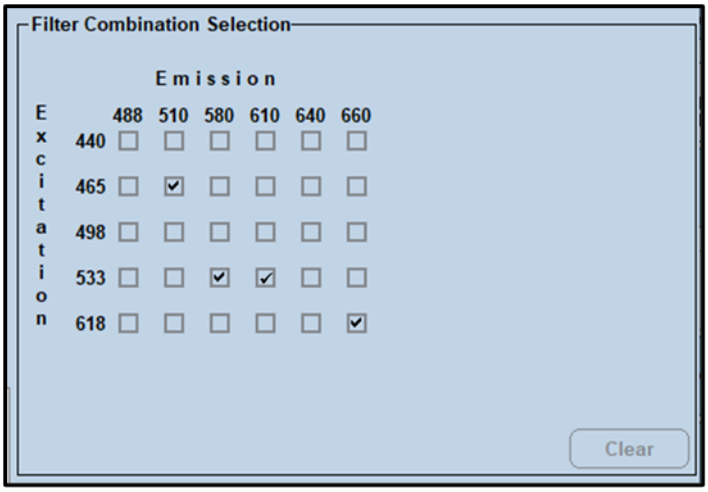
For other instruments

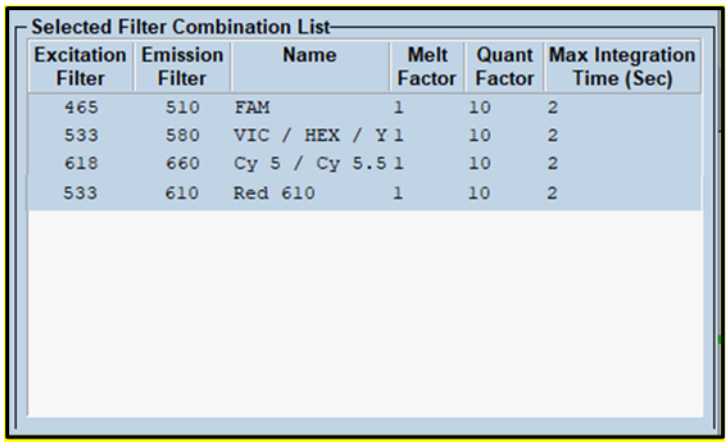
a) Enter the following amplification program:

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.

- When using the Roche® LightCycler 480 II you will need to create a custom detection format as directed in the LightCycler 480 instrument Operator's manual (2.1.1 Setting Detection Formats)
- The appropriate custom filter combination for the LightCycler 480 II is shown below:





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 CE IVD kit

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 Cy5 (618-660) and ROX (575-610) channels, has Cq of >37.37 in the FAM (465-510) channel of tube 1 only (the FAM channel of tube 2 should be free from amplification) and produces positive amplification in the VIC/HEX (533-580) channel (this is the detection of the genesig® Real-Time PCR SARS-CoV-2 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.

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/Yellow 555 (533-580)	
Cq <37.73	Cq (+)	Cq (+)	Cq (+) / (-)	SARS-CoV-2 Positive*
Cq <37.73	Cq (+)	Cq (-)	Cq (+) / (-)	SARS-CoV-2 Positive*
Cq <37.73	Cq (-)	Cq (+)	Cq (+) / (-)	SARS-CoV-2 Positive*
Cq <37.73	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 amplification of Cq <37.73 and/or Cy5 and/or ROX sample amplification in the ORF1ab/S/M testing indicate a SARS-CoV-2 positive sample. For the FluA/FluB/RSV test, all instances of FAM, Cy5, or ROX channel sample amplification indicate 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, ROX or Cy5 channels 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 IEC RNA 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 IEC RNA Cq < NEC IEC RNA 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 the accidental introduction of products from previous amplification reactions. Incorrect results could occur if either the clinical specimen or the real-time reagents used in the amplification step become contaminated by the accidental introduction of the 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 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 the 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 reagents. Do not substitute or mix reagents from different kits, lots or from other manufacturers.
- Change aerosol barrier pipette tips between all manual liquid transfers.
- During the 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.
- Worksurfaces, pipettes and centrifuges should be cleaned and decontaminated with cleaning products (e.g.,10% bleach, ethanol, DNA/RNA remover) to minimise the risk of nucleic acid contamination.
- RNA samples should be maintained on a cold block of ice during the 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 the 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 upper respiratory specimens 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 upper respiratory specimens

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. The 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:

SARS-CoV-2 target: Bio-Rad CFX Opus

Contrivance level in the PCR reaction (copies/rxn)	FAM (ORF1ab)		M gene (ROX)		S gene (Cy5)	
	Detection rate (%)	Mean Cq (SD)	Detection rate (%)	Mean Cq (SD)	Detect-ion rate (%)	Mean Cq (SD)
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)

Flu A target: Bio-Rad CFX Opus

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

Flu B target: Bio-Rad CFX Opus

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

RSV target: Bio-Rad CFX Opus

Contrivance level (copies/rxn)	Detection rate (%)	ROX Mean Cq (SD)
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 CE IVD kit detects as few 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 were 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 Limit of Blank

The Limit of Blank (LoB) is the highest apparent analyte concentration that is expected to be detected in any channel only when replicates of blank sample containing no analyte are tested. Positive amplification of Cq higher than the LoB in such channels will be concluded as negative. The LoB was determined by analysing 30 negative samples each in two batches of both tubes of the genesig® Real-Time PCR SARS-CoV-2 Winterplex CE IVD kit and the Cq values generated were used to determine what level of amplification could be considered insignificant. The LoB of the assay is 37.73 Cq in the FAM channel of tube 1 (SARS-CoV-2, ORF1ab target) and any Cq higher than this value in this channel only should be concluded as negative. There is no LoB applied to any other target in either tube.

9.1.3 Analytical sensitivity with alternative instruments: genesig q32 and Roche LightCycler 480 II

The SARS-CoV-2 targets are the same as found in the genesig® Real-Time PCR COVID-19 3G CE IVD kit, 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 genesig® Real-Time PCR SARS-CoV-2 Winterplex assay was validated with the Applied Biosystems 7500 instrument, and data on validation of the revised version of the kit on an Applied Biosystems platform will be included in a future iteration of this IFU. We have validated the revised assay for use on the Bio-Rad CFX Opus (see section 9.1.1), the genesig q32 Real-time PCR instrument and the Roche LightCycler 480 II. Upper respiratory specimens 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:

Flu A target: genesig q32 instrument

Concentration (copies/rxn)	Total Replicates	Detection Rate (%)	Mean HEX Cq (SD)	Mean FAM Cq (SD)
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)

Flu B target: genesig q32 instrument

Concentration (copies/rxn)	Total Replicates	Detection Rate (%)	Mean HEX Cq (SD)	Mean Cy5 Cq (SD)
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)

RSV target: genesig q32 instrument

Concentration (copies/rxn)	Total Replicates	Detection Rate (%)	Mean HEX Cq (SD)	Mean ROX Cq (SD)
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)

Flu A target: LightCycler 480 II instrument

Concentration (copies/rxn)	Total Replicates	Detection Rate (%)	Mean HEX Cq (SD)	Mean FAM Cq (SD)
3	20	95	22.05 (0.09)	38.16 (1.10)
2	20	85	21.75 (0.08)	38.53 (0.95)
1	20	60	21.66 (0.05)	38.97 (0.90)

Flu B target: LightCycler 480 II instrument

Concentration (copies/rxn)	Total Replicates	Detection Rate (%)	Mean HEX Cq (SD)	Mean Cy5 Cq (SD)
5	20	100	20.54 (0.27)	33.85 (0.53)
3	20	90	22.15 (0.21)	36.36 (1.08)
1	20	100	20.38 (0.34)	35.03 (0.45)

RSV target: LightCycler 480 II instrument

Concentration (copies/rxn)	Total Replicates	Detection Rate (%)	Mean HEX Cq (SD)	Mean ROX Cq (SD)
10	20	100	22.14 (0.11)	35.19 (0.44)
5	20	100	20.81 (0.09)	36.35 (0.78)
3	20	95	21.57 (0.07)	37.14 (1.03)

For Influenza A, using the genesig® q32, although no contrivance levels tested produced ≥95% positive amplification, the lowest contrivance level tested where ≥90% of samples amplified was 2 copies/ reaction. Therefore, the LoD_{90} using the genesig® q32 is 2 copies/reaction for synthetic Influenza A template. For the LightCycler, the LoD_{95} is 3 copies/reaction.

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 using the CFX Opus Real-Time PCR System. The LoD_{95} using the LightCycler is 3 copies/reaction for synthetic Influenza B RNA template.

Finally, for RSV-A and RSV-B, the lowest contrivance level tested where ≥95% of samples showed positive amplification was 3 copies/reaction using both the genesig® q32 and the LightCycler. This matches the verification LoD reported when using the CFX Opus Real-Time PCR System.

9.1.4 Analytical sensitivity with alternative instruments: Qiagen Rotor-Gene Q 5-plex

A further study with the Qiagen Rotor-Gene Q 5-plex (RGQ) established the LoD for both components of the genesig® Real-Time PCR SARS-CoV-2 Winterplex CE IVD kit. Heat-inactivated SARS-CoV-2 whole genome from ATCC was used to establish LoD with tube 1. RNA templates for Influenza A and Influenza B were obtained from Twist Bioscience, while RNA for RSV A & B was purchased from ATCC. Upper respiratory samples from negative donors were collected and stored in VTM and contrived with the relevant RNA prior to extraction using the exsig Mag Extraction System on the KingFisher Flex extraction platform. At least 20 replicates at each contrivance level were tested, and results are shown below:

SARS-CoV-2 target: Qiagen Rotor-Gene Q 5-plex

Contrivance level in the PCR reaction (copies/rxn)	Overall detection rate	FAM (ORF1ab)		M gene (ROX)		S gene (Cy5)	
		Detection rate (%)	Mean Cq (SD)	Detection rate (%)	Mean Cq (SD)	Detection rate (%)	Mean Cq (SD)
6	100%	5	36.61 (N/A)	10	36.73 (0.32)	100	30.42 (0.34)
5	100%	75	36.59 (0.69)	35	36.95 (0.87)	100	26.94 (0.63)
4	87.5%	35	36.75 (0.61)	30	38.07 (1.96)	85	31.47 (5.13)
3	83.3%	15	37.21 (0.20)	25	40.13 (2.47)	60	38.56 (1.51)
2	68.4%	20	36.77 (0.74)	30	37.51 (0.81)	50	37.92 (1.60)

Flu A target: Qiagen Rotor-Gene Q 5-plex

Concentration (copies/rxn)	Total Replicates	Detection Rate (%)	Mean HEX Cq (SD)	Mean FAM Cq (SD)
4	20	100%	30.93 (0.26)	36.08 (0.78)
3	20	95%	30.72 (0.28)	35.79 (0.87)
2	20	75%	30.90 (0.42)	36.88 (1.11)

Flu B target: Qiagen Rotor-Gene Q 5-plex

Concentration (copies/rxn)	Total Replicates	Detection Rate (%)	Mean HEX Cq (SD)	Mean Cy5 Cq (SD)
4	20	100%	28.01 (0.67)	36.52 (0.86)
3	20	100%	26.26 (4.85)	36.47 (0.60)
2	20	95%	28.20 (0.60)	37.77 (1.26)

RSV target: Qiagen Rotor-Gene Q 5-plex

Concentration (copies/rxn)	Total Replicates	Detection Rate (%)	Mean HEX Cq (SD)	Mean ROX Cq (SD)
5	20	100%	27.00 (0.82)	34.78 (0.84)
4	20	95%	28.32 (0.94)	35.28 (1.14)
3	20	100%	28.77 (0.45)	35.23 (1.07)
2	20	90%	30.52 (0.28)	35.77 (0.99)

To summarise, the LoD for the SARS-CoV-2 target is 5 copies in the reaction, and the respective LoDs for Flu A, Flu B and RSV are 3, 2, and 3 copies respectively.

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 CE IVD kit. 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 CE IVD kit. The in-silico analysis also evaluated assay inclusivity.

9.2.1 Latest in silico Specificity Analysis

To ensure the COVID-19 primer/probe remains 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 8th of June 2022, in silico analysis confirms the COVID-19 assay primers and probe still show 99.8%, 99.7% and 99.9% detection with the 9,859,534, 9,910,130 and 9,195,923 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 (19,911 sequences from the previous five years analysed in EpiFlu on 12th May 2022), Flu B (16,009 sequences from the previous five years analysed in EpiFlu on 10th May 2022) and RSV (all 3,308 sequences analysed from EpiRSV on 11th May 2022).

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:
NATtrol™ 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 CE IVD kit. The results of the *in vitro* cross-reactivity testing are presented below:

Sample number	Panel member	genesig® Real-Time PCR SARS-CoV-2 Winterplex assay – SARS-CoV-2 tube			
		FAM (ORF1ab)	HEX/VIC (IEC)	ROX (M gene)	Cy5 (S gene)
1	*A.baumannii*	N/A	25.5	N/A	N/A
2	*E.cloacae*	N/A	21.5	N/A	N/A
3	*E. coli*	N/A	22.4	N/A	N/A
4	*H.influenzae*	N/A	24.5	N/A	N/A
5	*K.oxytoca*	N/A	22.7	N/A	N/A
6	*P.aeruginosa*	N/A	23.3	N/A	N/A
7	*P.mirabilis*	N/A	23.9	N/A	N/A
8	*S.agalactiae*	N/A	23.8	N/A	N/A
9	*S.marcescens*	N/A	19.6	N/A	N/A
10	*S.pneumoniae*	N/A	24.0	N/A	N/A
11	*S.pyogenes*	N/A	24.9	N/A	N/A
12	*K.pneumoniae*	N/A	24.3	N/A	N/A
13	*K.pneumoniae*	N/A	23.7	N/A	N/A
14	*K.pneumoniae*	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 NATtrol™ Pneumonia Verification Panel (NATPPQ-BIO) from ZeptoMetrix, NAtrolTM 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 CE IVD kit shown in bold type.

Sample identity	Panel member	Cq obtained			
		FAM (Flu A)	ROX (RSV)	Cy5 (Flu B)	HEX (IEC)
PCT 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	*C. pneumoniae* 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	*Mycoplasma pneumoniae* (M129)	n/a	n/a	n/a	27.39
8	Parainfluenza Type 1	n/a	n/a	n/a	27.06

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Product code: D00020
Issue Number: 5.03
Published Date: 29th March 2023

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 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	*Legionella pneumophila* (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				
	Mycoplasma pneumoniae				
	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				
	Legionella pneumophila				
	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 CE IVD kit 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 the genesig® Real-Time PCR SARS-CoV-2 Winterplex CE IVD kit.

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® Real-Time PCR COVID-19 3G CE IVD kit (see the 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 Assay 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

Upper respiratory specimens 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 were 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, and the coefficient of variance (CV) used to determine variation between sites, operators, instruments and days,. 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

		Inter operator Contrivance level (x LoD)			Inter instrument Contrivance level (x LoD)			Inter day Contrivance level (x LoD)		
Batch	Channel (target)	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 genesig® Real-Time PCR SARS-CoV-2 Winterplex CE IVD 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® Real-Time PCR

COVID-19 3G CE IVD kit, and the previous version of the genesig® Real-Time PCR SARS-CoV-2 Winterplex CE IVD kit, 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 CE IVD kit 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 genesig® Real-Time PCR SARS-CoV-2 Winterplex CE IVD kit 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

Samples were tested blind with genesig® Real-Time PCR SARS-CoV-2 Winterplex CE IVD kit and compared with the contrivance status to produce the percentage agreements.
Briefly, 60 negative for SARS-CoV-2 upper respiratory specimens 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:

Results for the blind contrivance accuracy study using genesig® Real-Time PCR COVID-19 3G CE IVD kit.

		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

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 of the genesig® Real-Time PCR SARS-CoV-2 Winterplex CE IVD kit, 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.

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

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

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

Agreement	Target			
	SARS-CoV-2	Flu A	Flu B	RSV
OPA	100%	100%	100%	100%
PPA	100%	100%	100%	100%
NPA	100%	100%	100%	100%

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

9.5. Interfering substances

This revised genesig® Real-Time PCR SARS-CoV-2 Winterplex CE IVD kit 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 is available in the Assay 2020 IFU (https://www.genesig.com/assets/files/Path_SARS_CoV_2_Winterplex_IFU_Issue_102.pdf) and the genesig® Real-Time PCR COVID-19 3G CE IVD kit (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® Real-Time PCR COVID-19 3G CE IVD kit. 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® Real-Time PCR COVID-19 3G CE IVD kit, which has an identical formulation to the SARS-CoV-2 target tube of the genesig® Real-Time PCR SARS-CoV-2 Winterplex CE IVD kit.

This Clinical Performance Study was conducted at The Scientists Laboratory (TSL) Ltd, London. This study compared the genesig® Real-Time PCR COVID-19 3G CE IVD kit assay to a composite reference standard consisting of the following component tests:
- VIASURE SARS-CoV-2 Real-Time PCR Detection Kit
- genesig® Real-Time PCR Coronavirus (COVID-19) CE-IVD

Agreement between the component tests was considered to represent the true diagnostic status of a sample. If the two-component tests were not in accordance, the diagnostic status of the sample was determined to be inconclusive and the sample was excluded from the study.

The samples included in the study represented fresh or archived combined nasal/oropharyngeal samples. Fresh samples were assayed with genesig® Real-Time PCR Coronavirus (COVID-19) CE-IVD immediately after extraction with ThermoFisher MagMAX. The remaining eluates were frozen at -20 °C and thawed within 48 hours prior to analysis with genesig® Real-Time PCR COVID-19 3G CE IVD kit and VIASURE SARS-CoV-2 Real-Time PCR Detection Kit. Archived samples had been stored at -20°C in a transport medium for a maximum of 1 month. Following thawing, these samples were extracted with the ThermoFisher MagMAX kit and all three assays were performed on each specimen in parallel.
All assays were performed according to their respective instructions for use (IFUs):
- genesig® Real-Time PCR COVID-19 3G CE IVD kit Issue 4.0 (19th August 2021)*
- genesig® Real-Time PCR COVID-19 (CE IVD) IFU version 6.00 (11th June 2021)
- VIASURE SARS-CoV-2 Real-Time PCR Detection Kit IFU (IU-NCO212enes1120 rev.02 November 2020)
* genesig® Real-Time PCR COVID-19 3G CE IVD kit was analysed as per IFU Issue 8.0 (13th July 2022)

In total, 549 clinical specimens were submitted to the study. From these samples, 39 were excluded as the diagnostic status of the sample was determined to be inconclusive. For 10 samples, this was due to no result being available for one or more assays, and for the other 29 samples, there was discordance between the two assays of the composite reference standard.

Out of the 510 included samples:
- 190 positives were concordant between genesig® Real-Time PCR COVID-19 3G CE IVD kit and the composite reference standard.
- 317 negatives were concordant between genesig® Real-Time PCR COVID-19 3G CE IVD kit and the composite reference standard.
- 2 genesig® Real-Time PCR COVID-19 3G CE IVD kit -positives were discordant relative to the composite reference standard.
- 1 genesig® Real-Time PCR COVID-19 3G CE IVD kit-negative was discordant relative to the composite reference standard.

		Agreed result of VIASURE SARS-CoV-2 Real-Time PCR and genesig® Real-Time PCR COVID-19 assays		
		Positive	Negative	Total
genesig® Real-Time PCR COVID-19 3G CE IVD kit	Positive	190	2	192
	Negative	1	317	318
	Total	191	319	510

Clinical performance of genesig® Real-Time PCR COVID-19 3G CE IVD kit across a full viral range

	%	95% CI
Diagnostic Sensitivity	99.5%	97.13% - 99.99%
Diagnostic Specificity	99.4%	97.75% - 99.92%

From the positive samples, the FAM reading in the VIASURE SARS-CoV-2 assay can be used to divide the cohort of samples into low, medium, and high Cq ranges. Consequently, the sensitivity of the the genesig® Real-Time PCR COVID-19 3G CE IVD kit in each Cq range can also be determined:

Cq in comparator	% of samples	% sensitivity of genesig® Real-Time PCR COVID-19 3G CE IVD kit	95% CI
<25	22.99	100.0%	91.78% - 100.00%
≥25 to <30	25.67	100.0%	92.60% - 100.00%
≥30	51.34	99.0%	94.39% - 99.97%

Clinical performance of the Flu A/Flu B/RSV tube was evaluated with the previous genesig® Real-Time PCR SARS-CoV-2 Winterplex CE IVD kit, 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 assay (2020) and the new genesig® Real-Time PCR SARS-CoV-2 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 assay had previously (2020) consisted of the genesig® COVID-19 2G whilst the updated (2021) version has replaced it with the genesig® Real-Time PCR COVID-19 3G CE IVD kit.

As this shows that previous and updated genesig® Real-Time PCR SARS-CoV-2 Winterplex CE IVD kit have equivalent performance, data from the Performance Evaluation study at Tallaght University Hospital (Dublin, Ireland) can be considered here. A total of 132 results from respiratory samples were obtained and the Flu A/Flu B/RSV tube of the genesig® Real-Time PCR SARS-CoV-2 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	37	0	37
	Negative	1	93	94
	Total	38	93	131

		Comparator assay (TiBMolBiol)		
		Positive	Negative	Total
Influenza B	Positive	29	1	30
	Negative	0	100	100
	Total	29	101	130

		Comparator assay (TiBMolBiol)		
		Positive	Negative	Total
RSV	Positive	16	1	17
	Negative	0	113	113
	Total	16	114	130

Diagnostic Sensitivity and Specificity of the previous Assay 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® Real-Time PCR SARS-CoV-2 Winterplex CE IVD kit detects each target with a diagnostic sensitivity and diagnostic specificity >95% and that the previous and updated genesig® Real-Time PCR SARS-CoV-2 Winterplex CE IVD kits have equivalent clinical performance.

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: Primerdesign™ Ltd
- Address: York House, School Lane, Chandlers Ford, SO53 4DG
- Telephone number: +44 (0) 800 0156 494
- Website address: www.primerdesign.co.uk

13. Technical Support

For Technical support, please contact our dedicated technical support team on:
Phone: +44 (0) 23 80748830
Email: techsupport@primerdesign.co.uk

14. Symbols

Symbols	Meanings
	CE Mark
	EU Authorized Representative
	Contains sufficient for n tests
	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
	Storage temperature – Temperature Limit.