OSANTYS SARS-CoV-2 RT-qPCR Kit

INSTRUCTIONS FOR USE

Multiplex real-time RT-PCR test intended for the qualitative detection of SARS-CoV-2 RNA from unpurified and purified respiratory samples

REF **OS-COV2-100** Σ **100**

REF **OS-COV2-500** Σ **500**

Version 3.0

CE IVD

For *In Vitro* Diagnostic Use

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The customer is responsible for compliance with regulatory requirements that pertain to their procedures and uses of the instrument. The information in this guide is subject to change without notice.

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Revision History

Revision #	Effective Date	Summary of Revisions
1.0	2021, May	Original Instructions for Use
2.0	2022, January	Study of the Omicron variant impact on detection performance
3.0	2022, May	Additional performance details, impact of novel Omicron variants on detection performance and variant nomenclature update (30/05/2022)

Contents

Intended use

OSANTYS SARS-CoV-2 RT-qPCR Kit contains reagents and controls for a real-time reverse transcription-polymerase chain reaction (RT-qPCR) test intended for the qualitative detection of nucleic acid from SARS-CoV-2 in upper respiratory specimens such as nasopharyngeal (NP) and oropharyngeal (OP) swabs, and saliva from individuals suspected of COVID-19.

Results are for the identification of SARS-CoV-2 RNA. The SARS-CoV-2 RNA is generally detectable in upper respiratory specimens during the acute phase of infection. Positive results are indicative of the presence of SARS-CoV-2 RNA, but do not rule out bacterial infection or co-infection with other viruses. The agent detected may not be the definite cause of disease. Clinical correlation with patient history and other diagnostic information is necessary to determine patient infection status. Laboratories are required to report all positive results to the appropriate public health authorities.

Negative results do not preclude SARS-CoV-2 infection and should not be used as the sole basis for treatment or other patient management decisions. Negative results must be combined with clinical observations, patient history, and epidemiological information.

Testing with the OSANTYS SARS-CoV-2 RT-qPCR 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* diagnostics procedures.

Note: The following countries require the CE-marked *In Vitro* Diagnostics: Austria, Belgium, Bulgaria, Croatia, Cyprus, Czechia, Denmark, Estonia, Finland, France, Germany, Greece, Hungary, Ireland, Italy, Latvia, Lithuania, Luxembourg, Malta, Netherlands, Poland, Portugal, Romania, Slovakia, Slovenia, Spain, Sweden, UK, Norway, Iceland, Liechtenstein, Switzerland, Turkey.

Summary

An outbreak of pneumonia of unknown etiology in Wuhan City, Hubei Province, China was initially reported to WHO on December 31, 2019. Chinese authorities identified a novel coronavirus (SARS-CoV-2), which has resulted in millions of confirmed human infections globally. Cases of asymptomatic infection, mild illness, severe illness, and deaths have been reported.

OSANTYS SARS-CoV-2 RT-qPCR Kit is a molecular *in vitro* diagnostic test that aids in the detection and diagnosis of COVID-19 and is based on widely used nucleic acid amplification technology. The product contains oligonucleotide primers and dual-labeled double-quenched hydrolysis probes and control material used in RT-qPCR for the *in vitro* qualitative detection of SARS-CoV-2 RNA in respiratory specimens.

Test principle

The test is designed for specific detection of SARS-CoV-2 RNA using two primer/probe sets targeting two regions of the virus nucleocapsid (N) gene, N1 and N2. An additional primer/probe set to detect the human RNase P gene (RP) is also used to monitor the presence of PCR inhibitors in clinical specimen and evaluate specimen quality and RNA extraction to avoid a false negative result.

Starting material can be both purified nucleic acids from upper respiratory specimen, or directly unpurified respiratory specimen.

RNA is first reverse transcribed to cDNA and subsequently amplified in a one-step process using a real-time PCR instrument. In this process, each probe anneals to a specific target sequence located between the forward and reverse primers. During the extension phase of the PCR cycle, the 5' nuclease activity of Taq polymerase degrades the probe, causing the reporter dye to separate from the quencher dye, generating a fluorescent signal. With each cycle, additional reporter dye molecules are cleaved from their respective probes, increasing the fluorescence intensity. Fluorescence intensity is monitored at each PCR cycle by the real-time PCR instrument. The data are analyzed and interpreted by the analysis software used with the instrument.

Product description

The OSANTYS SARS-CoV-2 RT-qPCR Kit contains the following components:

Reagent Name//Part Number	***Description***	***Quantity & Volume***		***Storage***	***Shelf life[1]***
		100 reactions	***500 reactions***		
SARS-CoV-2 Primer/Probe Mix // OS-COV2-100-1	N1, N2 and RP Combined Primer/Probe Mix	750 µL/tube x 1	750 µL/tube x 5	-25°C to -15°C protected from light	12 months
One-Step RT-qPCR Enzyme Master Mix 2X // OS-COV2-100-2	reagents for RT-PCR including RT and Taq Polymerase	625 µL/tube x 2	625 µL/tube x 10	-25°C to -15°C	12 months
SARS-CoV-2 Positive Controls // OS-COV2-100-3	plasmids containing SARS-CoV-2 N gene and human RP gene	26 µL/tube x 1	26 µL/tube x 5	-25°C to -15°C	12 months
SARS-CoV-2 Negative Control // OS-COV2-100-4	DNase/RNase free water	100 µL/tube x 1	100 µL/tube x 5	-25°C to -15°C	12 months

[1]The shelf life of the kit is determined by the component with the shortest shelf life.

After 5 thaw / freeze cycles of the entire kit, no reduction in RT-qPCR performance was observed.

- SARS-CoV-2 Positive Control and RNase P Positive Control are used to monitor RT-qPCR reaction setup and reagent integrity.
- SARS-CoV-2 Negative Control is used to monitor cross-contamination during RNA extraction and reaction setup.

Symbols

	Contains reagents sufficient for <N> reactions
	Use by date
	In vitro diagnostic medical device
	Catalog number
	Batch number
	Material number
	Components
	Contents
	Number
	Temperature limitations
	Manufacturer
	Instructions for use
	Important note

Required materials not supplied

- Nucleic acid extraction system (if used): commercially available viral RNA extraction kits and automated extractors that have been qualified and validated for recovery and purity of viral RNA in NP and OP swabs, and in saliva can be used.
- Thermo-block for virus inactivation (if used)
- Saline solution or PBS to dilute saliva specimen
- Transport media: any transport media validated for virus conservation from nasopharyngeal swab, or saline solution can be used with nucleic acid purification. For **unpurified samples,** do not use virus inactivating media and media containing guanidine or phenol red.
- Real-time PCR instrument
- Laboratory freezers -25°C to -15°C
- Vortex mixer
- Racks for 1.5 mL microcentrifuge tubes and 96-well plates or strip tubes
- Microcentrifuge for tubes
- Centrifuge for 96-well plates
- Single channel adjustable micropipettes (10 µL, 20 µL, 200 µL and 1000 µL)
- Multichannel adjustable micropipettes (20 µL or 200 µL)
- Sterile aerosol barrier (filtered) pipette tips
- Disposable pipetting reservoirs (VWR catalog n. 613-1179 or equivalent)
- 1.5 mL microcentrifuge DNase/RNase free tubes
- PCR optical reaction plates or tubes:
  - 0.1 or 0.2 ml PCR optical 96-well reaction plate
  - optical adhesive film
  - adhesive film applicator

or

  - 0.1 or 0.2 ml PCR strip tubes
  - optical 8-cap Strips
- Detergent/disinfectant (Surfa'Safe Premium Anios, or equivalent)
- Reagents for DNA and RNase removal (DNA-ExitusPlus, RNase-ExitusPlus or equivalent)
- Disposable powder-free gloves and surgical gowns

Warnings and precautions

- Handle all specimens and controls as if infectious in accordance with safe laboratory procedures. Refer to 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.
- Specimen processing should be performed in accordance with national biological safety regulations.
- Perform all manipulations of live virus samples within a Class II (or higher) biological safety cabinet (BSC).
- Heat-inactivation of live virus at 56°C for 30 minutes is highly recommended if you use unpurified specimen as starting material. Heat inactivation does not affect the detection performance of the kit.
- Use personal protective equipment (PPE) such as (but not limited to) gloves, eye protection, and lab coats when handling specimen and while performing this assay and handling materials including samples, reagents, pipettes, and other equipment and reagents.
- Always use pipette tips with aerosol barriers. Tips that are used must be sterile and free from DNases and RNases.
- Use separate areas for the preparation of patient samples and controls to prevent false positive results. Samples and reagents must be handled under a laminar airflow hood or PCR hood.
- Do not eat, drink, smoke, apply cosmetics or handle contact lenses in areas where reagents and human specimens are handled.
- Performance characteristics have been determined with human upper respiratory specimens from human patients with signs and symptoms of respiratory infection.
- Modifications to assay reagents, assay protocol, or instrumentation are not permitted, and are in violation of the *In Vitro* Diagnostic Directive 98/79/EC.
- Do not mix or exchange components from different kit batches.
- Do not use the kit past the expiration date.
- Dispose of waste in compliance with the local regulations.
- Safety Data Sheets are available upon request.
- The quality of the sample and sample preparation (purified RNA) may influence the quality of the RT-qPCR test. It is highly recommended to process the sample as soon as possible after collection and store it refrigerated (+ 2°C to + 8°C) before processing. Freezing at -20°C can cause significant degradation of viral RNA.
- 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). Workflow in the laboratory should proceed in a unidirectional manner.

- Maintain separate areas for assay setup and handling of samples.
- 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. Proper aseptic technique should always be used when working with nucleic acids.
- Maintain separate, dedicated equipment (e.g., pipettes, microcentrifuges) and supplies (e.g., microcentrifuge tubes, pipette tips) for assay setup and handling of extracted nucleic acids and unpurified samples.
- Wear a clean lab coat and powder-free disposable gloves (not previously worn) when setting up assays.
- Change gloves between samples and whenever contamination is suspected.
- Keep reagent and reaction tubes capped or covered as much as possible.
- Reagents can be thawed at room temperature and maintained at room temperature at all times during preparation and use.
- Work surfaces, pipettes, centrifuges and other equipment should be cleaned and decontaminated with cleaning products such as Surfa'Safe Premium Anios, DNA-ExitusPlus and RNase-ExitusPlus to minimize risk of nucleic acid contamination.

- Purified RNA samples and unpurified specimen can be maintained at room temperature during assay set-up. It is highly recommended to store them refrigerated (+2°C - +8°C) before use to ensure stability.
- Dispose of unused kit reagents and human specimens according to local regulations.

Assay limitations

- This assay is intended to be used for *in vitro* diagnostic purposes only. Follow good laboratory practices and all precautions and established guidelines to avoid cross-contamination between samples.
- Performance of the OSANTYS SARS-CoV-2 RT-qPCR Kit has only been established in upper respiratory specimens such as nasopharyngeal or oropharyngeal swabs, and saliva. Other specimen types have not been evaluated and should not be tested with this assay.
- Samples must be collected, transported, and stored using appropriate procedures and conditions. Improper collection, transport, or storage of specimens may hinder the ability of the assay to detect the target sequences.

- This kit uses purified and unpurified RNA as a sample for the analysis. The quality of the samples and of the RNA recovered from biological samples is essential for the quality of the results generated with this kit.

Negative results do not preclude SARS-CoV-2 infection and should not be used as the sole basis for treatment or other patient management decisions. Collection of multiple specimens (types and time points) from the same patient may be necessary to detect the virus.

- **False-negative results** may arise from:
  - Improper sample collection, transport or handling.
  - Degradation of the SARS-CoV-2 RNA during shipping/storage.
  - Specimen collection after SARS-CoV-2 RNA can no longer be found in the specimen matrix.
  - Using poor or unauthorized extraction methods.
  - The presence of RT-PCR inhibitors.
  - inadequate numbers of organisms present in the specimen.
  - Mutation in the SARS-CoV-2 virus.
  - Failure to follow instructions for use.
- **False-positive results** may arise from:
  - Cross contamination during specimen handling or preparation.
  - Cross contamination between patient samples.
  - Specimen mix-up.
  - RNA contamination during product handling.
- The impacts of vaccines, antiviral therapeutics, antibiotics, chemotherapeutic or immune
- suppressant drugs have not been evaluated.
- This test cannot rule out diseases caused by other bacterial or viral pathogens.

Equipment preparation

Clean and decontaminate all work surfaces, pipettes, centrifuges, and other equipment prior to use. Decontamination agents should be used to minimize the risk of nucleic acid contamination and degradation.

Preparation of unpurified respiratory specimen

This kit can be used with unpurified NP and OP swabs collected in the following transport media:

- Saline solution
- ∑-Virocult (MWE)
- ∑-VCM (MWE)
- ∑-Transwab (MWE)
- DeltaSwab liquid amies (Deltalab)

- Fecal Transwab – liquid Cary Blair (MWE)

- Do not use inactivating media, any chemical inactivation methods, nor media containing guanidine or phenol red, as they would inhibit the RT-qPCR reaction.
- Heat inactivation at 56°C for 30 minutes is recommended for safety purposes and does not affect the detection performance.

Nucleic acid extraction

Performance of the test is dependent upon the amount and quality of template RNA purified from human specimens. The following commercially available RNA extraction kit, and automated extractor and procedures have been qualified and validated for recovery and purity of RNA for use with the kit:

- **MGISP-NE32 Automated nucleic acid extractor and Nucleic acid extraction kit – 32 preps (MGI)** Catalog n. 100022606
  - Follow recommended manufacturer's instructions for NP and OP swabs.
  - For saliva specimen, add 50 µL saliva to 250 µl of saline solution and proceed to RNA extraction according to manufacturer's instructions. In the case of a highly viscous sample, a sample: saline solution/PBS ratio up to 1:10 can be used without significant loss of kit performance.

Other commercially available RNA extraction kits and automated extractors that have been qualified and validated for recovery and purity of viral RNA in NP and OP swabs, and in saliva can be used.

Guidelines for RT-qPCR

- Run the plate immediately after preparation. Failure to do so could result in degraded RNA samples.
- To prevent contamination, prepare reagents in a PCR workstation or equivalent amplicon-free area.
- Do not use the same pipette for reaction mix and RNA samples.
- Always use aerosol barrier pipette tips. Maintain an RNase-free environment.
- Protect Primer/Probe mix from light.
- For each run plate, include one negative control and one positive control.

Assay set up

Reaction Master Mix and plate set up

1) In the reagent set-up dedicate hood, place the SARS-CoV-2 Primer/Probe Mix and the One-Step RT-qPCR Enzyme Master Mix 2X tubes in a rack.

2) Thaw the reagents at room temperature and maintained them at room temperature during preparation.

3) Gently vortex the SARS-CoV-2 Primer/Probe Mix and mix the One-Step RT-qPCR Enzyme Master Mix by inversion 5 times.

4) Centrifuge reagents briefly to collect contents at the bottom of the tube.

5) Determine the number of reactions (N) to set up per assay. Include excess reaction mix for pipetting error. Use the following guide to determine N:

- If number of samples (n) including controls equals 1 through 24, then N = n + 1
- If number of samples (n) including controls is 25 through 48, then N = n + 2
- If number of samples (n) including controls is 49 through 72, then N = n + 3
- If number of samples (n) including controls is 73 through 96, then N = n + 4

6) For each run, in a 1.5 mL microcentrifuge tube combine the following components:

Component	Volume/Reaction
One-Step RT-qPCR Enzyme Master Mix 2X	N x 12.5 µL
SARS-CoV-2 Primer/Probe Mix	N x 7.5 µL
Total Volume	**N x 20.0 µL**

7) Mix reaction mixtures by pipetting up and down. ***Do not vortex***.

8) Centrifuge briefly to collect contents at the bottom of the tube.

9) Set up reaction strip tubes or plates in a 96-well rack.

10) Pipette 20 µL of the reaction mix into each well.

12) Prior to moving to the sample handling hood, prepare the No Template Control (NTC) reaction in the reagent set-up hood.

13) Pipette 5 µL of SARS-CoV-2 Negative Control into the NTC well in A1 position or before the first clinical sample. Seal the NTC well with a cap, or a part of an adhesive film.

14) Cover the entire reaction plate and move the reaction plate to the sample handling hood.

Sample addition

1) Vortex *purified* samples tubes for approximately 5 seconds at 2.000 rpm
2) Centrifuge briefly to collect contents at the bottom of the tube.

or

Allow *unpurified* refrigerated samples to reach room temperature, then vortex samples tubes for approximately 15 seconds at 2.000 rpm.

3) Carefully pipette 5.0 µL of each sample into each well. *Change tips after each addition.*
4) Pipette 5 µL of Positive Controls into the positive control well in position H12 or after the last clinical sample.
5) Seal strip tubes with strip caps, or plate with adhesive film.
6) Briefly centrifuge reaction tube strips or plate for 30 seconds.

Set up the real-time PCR instrument

1. Create the following thermal protocol:

   - Step 1/Reverse Transcription:
     52°, 5 min
   - Step 2/RT Inactivation-Polymerase Activation:
     95°, 10 sec
   - Step 3/PCR reaction:
     Denaturation: 95°C, 5 sec
     Anneal/Extension: 63.1°C, 30 sec (+ plate read)
     45 cycles

2. Select reporter dyes:

Target	Reporter Dye/Channel
N1	FAM/blue
N2	VIC/green
RP	Cy5/red

3. Indicate 25 µl as reaction volume per well.
4. Load the strip tubes or plate into the thermocycler and start the run.
5. After the instrument run is complete, analyze the data.

This kit has been validated for use with:

- CFX96™ Dx System (Bio-Rad) and CFX Dx Manager software version 3.1 (optical plates and tubes with white wells)
- QuantStudio 5 Dx Real-Time PCR System and QuantStudio Design and Analysis Desktop Software v1.5.1 (Thermo Fisher Scientific)

Any other Real-Time PCR system with channels for FAM, VIC and Cy5 fluorescence reading and not requiring ROX or other reference dye calibration, can be used with this kit.

Data analysis

Refer to your instrument manual for generating the amplification plot and Ct values for each sample, and define baseline threshold and setting.

Result interpretation

Expected performance of Controls included in the OSANTYS SARS-CoV-2 RT-qPCR Kit

Control Type	Used to Monitor	N1	N2	RP	Expected Ct Values
Positive	Substantial reagent failure including primer and probe integrity	+	+	+	< 40 Ct
Negative	Reagent and/or environmental contamination	-	-	-	None detected
Endogenous	Failure in lysis and extraction procedure, potential contamination during extraction	-	-	+	< 40 Ct

If any of the above controls do not exhibit the expected performance as described, the assay may have been set up and/or executed improperly, or reagent or equipment malfunction may have occurred. Invalidate the run and re-test.

Results interpretation for patient samples

N1	N2	RP	Result interpretation [a]	Report	Actions
Ct < 40	Ct < 40	±	SARS-CoV-2 detected	Positive for SARS-CoV-2	Report results to local health authority and sender
If only one of the two targets is positive		±	SARS-CoV-2 detected if Ct < 37	Positive for SARS-CoV-2	Report results to local health authority and sender
			Inconclusive if Ct > 37	Inconclusive	
Ct > 40 or ND	Ct > 40 or ND	Ct < 40	SARS-CoV-2 not detected	Not Detected	Report results to sender. Consider testing for other respiratory viruses [b]
-	-	-	Invalid Result	Invalid	Repeat extraction and RT-qPCR. If the repeated result remains invalid, consider collecting a new specimen from the patient.

ND = not detected

[a] Laboratories should report their diagnostic result as appropriate and in compliance with their specific reporting system.

[b] Optimum specimen types and timing for peak viral levels during infections caused by SARS-CoV-2 have not been determined. Collection of multiple specimens from the same patient may be necessary to detect the virus. The possibility of a false negative result should especially be considered if the patient's recent exposures or clinical presentation suggest that SARS-CoV-2 infection is possible, and diagnostic tests for other causes of illness (e.g., other respiratory illness) are negative. If SARS-CoV-2 infection is still suspected, re-testing should be considered in consultation with public health authorities.

If a laboratory obtains unexpected results for assay controls, or if inconclusive or invalid results are obtained and cannot be resolved through the recommended re-testing, please contact Osantys for consultation and possible specimen referral.

RNase P (Endogenous Control)

- All clinical samples should exhibit fluorescence signal for the RP < 40 Ct, thus indicating the presence of the human RNase P gene. Failure to detect RNase P in any clinical specimens may indicate:

- Improper extraction of nucleic acid from clinical materials resulting in loss of RNA and/or RNA degradation.
- Absence of sufficient human cellular material due to poor collection or loss of specimen integrity.
- Improper assay set up and execution.
- Reagent or equipment malfunction.

- If the RP assay does not produce a positive result for clinical samples, interpret as follows:

- If the SARS-CoV-2 N1 and N2 are positive even in the absence of a positive RP, the result should be considered valid.

It is possible that some samples may fail to exhibit RP growth curves due to low cell numbers in the original clinical sample, or reagent competition. A negative RP signal does not preclude the presence of SARS-CoV-2 virus RNA in a clinical sample.

- If N1, N2 *AND* RP are negative for the specimen, the result should be considered invalid for the specimen.

If residual specimen is available, repeat the extraction procedure and repeat the test.
If all markers remain negative after re-test, report the results as invalid and a new specimen should be collected if possible.

N1 and N2 SARS-CoV-2 Markers

- When all controls exhibit the expected performance, a specimen is considered ***negative*** if both N1 AND N2 signals are not detected or > 40 Ct AND the RP signal is < 40 Ct.
- When all controls exhibit the expected performance, a specimen is considered ***positive*** for SARS-CoV-2 if both N1 AND N2 signals are < 40 Ct. The RP may or may not be positive as described above, but the result is still valid.
- When all controls exhibit the expected performance, and only one of the SARS-CoV-2 markers (N1 OR N2 but not both markers) shows a signal < 40 Ct, interpret as follows:
    - If N1 or N2 Ct is < 37, a specimen is considered ***positive***. A mutation affecting the other probe could have impacted the detection of the signal.
    - If N1 or N2 Ct is > 37, the result is ***inconclusive***. The sample should be retested. If the same result is obtained, report the inconclusive result and proceed with a new specimen collection.
- When all controls exhibit the expected performance, and Ct for N1, N2 AND the RP markers are not detected, the result is ***invalid***. The sample should be retested. If the re-tested sample is negative for all markers, the result is invalid and collection of a new specimen from the patient should be considered.

Quality Control

- Quality control requirements must be performed in conformance with local regulations or accreditation requirements and the user's laboratory's standard quality control procedures. For further guidance on appropriate quality control practices, refer to refer to your governmental laboratory accreditation system.
- Quality control procedures are intended to monitor reagent and assay performance.
- Always include a negative template control (NTC) and the appropriate SARS-CoV-2 positive control in each amplification run.
- All clinical samples should be tested for human RP gene to control for specimen quality and extraction.

Performance Characteristics

The analytical and clinical performance of the OSANTYS SARS-CoV-2 RT-qPCR Kit was evaluated by comparing performance according to specimen type, determining limit of detection (LoD), characterizing specificity, cross-reactivity and interfering substances, as described in the following sections.

Analytical performance

Performance evaluation between purified and non-purified specimen

To compare kit performance according to specimen type (purified RNA or unpurified respiratory specimen – direct RT-qPCR), NP swabs previously tested positive or negative with the Novel Coronavirus (2019-nCoV) Nucleic Acid Diagnostic Kit (PCR-Fluorescence Probing) – Sansure Biotech, were tested following or not RNA extraction.

- 84 positive NP specimen
- 118 negative NP specimen

Table 1a. Performance evaluation between purified and unpurified positive specimen

	N1			N2			RP		
Sample	**Purified RNA**	**Direct RT-qPCR**	**Δ Ct**	**Purified RNA**	**Direct RT-qPCR**	**Δ Ct**	**Purified RNA**	**Direct RT-qPCR**	**Δ Ct**
157*	15,04	16,70	**-1,66**	14,03	17,17	**-3,14**	25,80	26,87	**-1,07**
60*	15,45	17,84	**-2,39**	14,53	18,06	**-3,53**	28,21	32,25	**-4,04**
137*	16,17	24,88	**-8,71**	15,32	24,03	**-8,71**	26,93	30,19	**-3,26**
123*	16,32	19,69	**-3,37**	15,61	19,94	**-4,33**	23,75	25,31	**-1,56**
61*	17,31	20,76	**0,78**	16,53	21,10	**-0,34**	27,19	29,04	**-1,85**
80*	17,53	21,84	**-4,31**	16,65	21,40	**-4,75**	28,93	32,73	**-3,80**
132*	17,74	22,02	**-4,28**	16,74	21,94	**-5,20**	27,55	30,22	**-2,67**
134*	17,89	25,46	**-7,57**	17,11	25,04	**-7,93**	29,58	33,13	**-3,55**
20*	17,93	20,19	**-2,26**	17,18	20,57	**-3,39**	26,77	28,35	**-1,58**
22*	18,11	20,87	**-2,76**	17,4	21,61	**-4,21**	27,83	29,91	**-2,08**
70*	18,19	23,26	**-5,07**	17,08	23,95	**-6,87**	27,53	32,29	**-4,76**
133*	18,77	22,12	**-3,35**	17,98	22,30	**-4,32**	25,80	24,76	**1,04**
76*	19,01	23,21	**-4,20**	17,96	23,82	**-5,86**	29,73	31,64	**-1,91**
19*	19,09	21,16	**-2,07**	18,11	21,28	**-3,17**	27,3	29,86	**-2,56**
69*	19,61	22,67	**-3,06**	18,47	22,79	**-4,32**	27,86	31,90	**-4,04**
130*	19,81	23,31	**-3,50**	19,11	23,82	**-4,71**	26,06	27,14	**-1,08**
144*	19,84	23,64	**-3,80**	19,25	23,72	**-4,47**	27,67	31,37	**-3,70**
56*	20,06	22,76	**-2,70**	19,47	23,30	**-3,83**	27,03	30,10	**-3,07**
26*	20,19	21,99	**-1,80**	19,51	22,52	**-3,01**	29,83	32,17	**-2,34**

	N1			N2			RP		
Sample	Purified RNA	Direct RT-qPCR	ΔCt	Purified RNA	Direct RT-qPCR	Δ Ct	Purified RNA	Direct RT-qPCR	ΔCt
128*	20,78	25,37	**-4,59**	20,24	25,11	**-4,87**	28,08	32,10	**-4,02**
94*	21,56	26,81	**-5,25**	20,61	26,23	**-5,62**	28,70	32,19	**-3,49**
4*	21,65	25,75	**-4,10**	21,29	26,1	**-4,81**	29,28	31,86	**-2,58**
63*	21,65	22,36	**-0,71**	20,84	23,62	**-2,78**	27,59	30,32	**-2,73**
16*	21,89	25,74	**-3,85**	21,3	25,63	**-4,33**	28,61	30,78	**-2,17**
142*	21,95	26,00	**-4,05**	21,03	25,88	**-4,85**	26,23	28,56	**-2,33**
140*	22,09	24,01	**-1,92**	21,40	24,16	**-2,76**	26,68	28,85	**-2,17**
122*	22,22	24,88	**-2,66**	21,89	25,14	**-3,25**	27,76	30,15	**-2,39**
152*	22,48	24,24	**-1,76**	22,30	24,88	**-2,58**	30,01	32,45	**-2,44**
149*	22,60	26,78	**-4,18**	21,76	26,43	**-4,67**	29,66	33,33	**-3,67**
155*	23,08	24,78	**-1,70**	22,65	25,51	**-2,86**	28,03	30,58	**-2,55**
150*	23,14	27,00	**-3,86**	22,28	26,88	**-4,60**	23,57	24,86	**-1,29**
85*	23,20	27,71	**-4,51**	22,67	27,98	**-5,31**	29,03	31,81	**-2,78**
12*	23,55	26,54	**-2,99**	23,83	27,11	**-3,28**	26,18	26,85	**-0,67**
112*	23,74	26,44	**-2,70**	22,92	26,21	**-3,29**	28,06	30,96	**-2,90**
131*	23,83	31,80	**-7,97**	23,09	31,51	**-8,42**	25,03	28,31	**-3,28**
88*	23,86	27,75	**-3,89**	22,88	28,31	**-5,43**	26,99	32,30	**-5,31**
120*	23,91	28,70	**-4,79**	23,25	27,53	**-4,28**	27,64	30,79	**-3,15**
23*	23,94	25,32	**-1,38**	23,24	25,66	**-2,42**	29	31,41	**-2,41**
127*	24,87	29,33	**-4,46**	24,60	29,13	**-4,53**	26,56	28,53	**-1,97**
64*	24,93	26,07	**-1,14**	24,81	30,75	**-5,94**	30,3	30,97	**-0,67**
110*	25,12	30,17	**-5,05**	24,19	30,02	**-5,83**	26,67	28,53	**-1,86**
147*	25,77	33,37	**-7,60**	24,98	33,08	**-8,10**	27,72	30,57	**-2,85**
108*	26,16	27,81	**-1,65**	24,69	26,86	**-2,17**	28,68	30,83	**-2,15**
10033*	26,20	27,95	**-1,75**	25,57	28,01	**-2,44**	27,12	30,84	**-3,72**
111*	26,46	32,00	**-5,54**	35,72	31,86	**3,86**	26,41	30,43	**-4,02**
62*	26,58	27,6	**-1,02**	25,97	27,59	**-1,62**	25,49	31,75	**-6,26**
139*	26,62	29,69	**-3,07**	25,87	28,51	**-2,64**	28,58	31,73	**-3,15**
121*	26,77	29,64	**-2,87**	25,66	28,88	**-3,22**	27,13	30,44	**-3,31**
117*	27,16	29,13	**-1,97**	26,47	28,81	**-2,34**	25,41	29,20	**-3,79**
2*	27,31	30,77	**-3,46**	26,78	30,96	**-4,18**	28,63	32,56	**-3,93**
138*	27,38	31,11	**-3,73**	26,82	29,96	**-3,14**	30,07	33,39	**-3,32**
146*	28,33	30,11	**-1,78**	27,66	29,85	**-2,19**	27,09	29,15	**-2,06**
151*	28,76	33,41	**-4,65**	27,80	33,08	**-5,28**	29,01	31,76	**-2,75**
125*	28,81	32,79	**-3,98**	28,03	32,15	**-4,12**	27,83	30,40	**-2,57**
136*	28,86	31,44	**-2,58**	28,19	31,08	**-2,89**	29,61	31,73	**-2,12**
154*	29,23	32,78	**-3,55**	28,14	31,91	**-3,77**	24,3	26,32	**-2,02**
153*	29,38	35,78	**-6,40**	28,42	35,12	**-6,70**	26,99	30,74	**-3,75**
74*	29,75	32,89	**-3,14**	29,01	34,19	**-5,18**	25,73	25,77	**-0,04**
96*	30,06	36,60	**-6,54**	29,16	35,93	**-6,77**	27,68	30,48	**-2,80**
148*	30,75	ND	-	29,95	37,87	**-7,92**	29,89	35,58	**-5,69**

	N1			N2			RP		
Sample	**Purified RNA**	**Direct RT-qPCR**	**ΔCt**	**Purified RNA**	**Direct RT-qPCR**	**ΔCt**	**Purified RNA**	**Direct RT-qPCR**	**ΔCt**
25*	31,04	33,18	**-2,14**	29,99	31,75	**-1,76**	29,34	31,36	**-2,02**
1*	31,13	34,23	**-3,10**	30,55	34,33	**-3,78**	28,7	30,89	**-2,19**
48*	31,20	34,04	**-2,84**	30,70	33,86	**-3,16**	29,65	31,31	**-1,66**
8*	31,2	33,74	**-2,54**	30,02	33,53	**-3,51**	30,22	33,06	**-2,84**
10*	31,41	35,39	**-3,98**	30,98	34,8	**-3,82**	27,72	29,46	**-1,74**
156*	31,73	34,08	**-2,35**	30,9	34,71	**-3,81**	28,53	32,35	**-3,82**
89*	31,89	ND	-	31,30	ND	-	26,00	31,23	**-5,23**
105*	31,91	34,48	**-2,57**	31,55	36,32	**-4,77**	23,65	24,54	**-0,89**
145*	31,93	35,49	**-3,56**	31,01	33,18	**-2,17**	28,52	32,43	**-3,91**
124*	32,03	34,13	**-2,10**	31,5	34,07	**-2,57**	25,57	29,79	**-4,22**
143*	32,17	35,76	**-3,59**	31,76	36,83	**-5,07**	25,26	27,70	**-2,44**
103*	32,86	33,49	**-0,63**	32,21	32,93	**-0,72**	26,42	27,76	**-1,34**
55*	32,99	34,21	**-1,22**	32,08	34,02	**-1,94**	25,57	27,85	**-2,28**
54*	33,01	36,71	**-3,70**	31,94	35,91	**-3,97**	28,71	31,74	**-3,03**
129*	33,29	ND	-	32,32	ND	-	27,06	30,25	**-3,19**
67*	33,47	34,58	**-1,11**	32,74	34,53	**-1,79**	26,81	26,76	**0,05**
104*	33,85	32,75	**1,10**	28,91	32,45	**-3,54**	27,70	29,89	**-2,19**
126*	35,79	ND	-	35,32	ND	-	28,85	31,26	**-2,41**
97*	36,40	36,70	**-0,30**	35,68	36,73	**-1,05**	27,62	30,93	**-3,31**
107*	38,11	38,12	**-0,01**	35,69	41,09	**-5,40**	32,90	35,75	**-2,85**
92*	38,16	ND	-	36,62	ND	-	25,89	27,79	**-1,90**
51*	ND	ND	-	38,20	ND	-	28,76	30,48	**-1,72**
141*	ND	ND	-	ND	37,80	-	31,53	33,42	**-1,89**
135*	ND	ND	-	37,38	ND	-	27,85	32,46	**-4,61**
Δ Ct mean			**-3,20**			**-3,98**			**-2,70**

**Samples stored frozen at -20°C for more than 10 days. Degradation may have occurred*

ND = not detected

A variable ΔCt is observed between purified and unpurified specimen, independent of viral charge. For Ct > 35, RNA extraction increased the possibility of SARS-CoV-2 detection.

Table 1b. Performance evaluation between purified and unpurified negative specimen

Results	Purified RNA	DIRECT RT-qPCR
Negative/Expected Negative	115/118	116/118
Positive*/Expected Negative	**3**/118	**2**/118

	N1		N2		RP	
*Sample	Purified RNA	Direct RT-qPCR	Purified RNA	Direct RT-qPCR	Purified RNA	Direct RT-qPCR
20023	35,77	ND	35,77	ND	30,52	33,60
20024	33,92	37,93	34,09	36,30	26,67	31,67
10162	37,21	ND	35,64	ND	29,65	32,50
10030	ND	ND	ND	37,94	26,48	29,73

Limit of detection (LoD)

The LoD study established the lowest SARS-CoV-2 concentration that can be detected by the OSANTYS SARS-CoV-2 RT-qPCR Kit in a particular specimen type at least 95% of time. The LoD was determined by limiting dilution studies.

1. Purified RNA from a validated positive NP swab was 10-fold serially diluted in validated transport media and then processed using the kit workflow for unpurified samples. 8 replicates were realized for each dilution. Quantified SARS-CoV-2 positive control was used to determine viral copy numbers. The LoD is indicated in the yellow case.

Table 2. LoD determination in unpurified NP swabs in *saline solution*

Targets	N1				N2			
RNA Concentration[1]	10^2	10^1	$10^{0.0}$	10^{-1}	10^2	10^1	$10^{0.0}$	10^{-1}
Positives/Total	8/8	8/8	8/8	7/8	8/8	8/8	8/8	7/8
Mean Ct[2]	34,95	37,4	37,03	37,05	34,75	37,74	36,14	37,23
Standard deviation (Ct)	0,329	0,732	0,881	0,680	0,694	1,832	0,676	1,217

1. Concentration is presented in RNA copies/reaction
2. Mean Ct reported for dilutions that are ≥ 95% positive. Calculations only include positive results. ND = not detected

Table 3. LoD determination in unpurified NP swabs *in Fecal Transwab – liquid Cary Blair (MWE)*

Targets	N1				N2			
RNA Concentration[1]	10^2	10^1	$10^{0.0}$	10^{-1}	10^2	10^1	$10^{0.0}$	10^{-1}
Positives/Total	8/8	8/8	6/8	3/8	8/8	8/8	2/8	1/8
Mean Ct[2]	33,91	36,44	38,15	38,99	34,23	38,65	45,29	49
Standard Deviation (Ct)	0,491	0,825	0,972	0,199	0,535	2,197	7,41	-

1. Concentration is presented in RNA copies/reaction
2. Mean Ct reported for dilutions that are ≥ 95% positive. Calculations only include positive results. ND = not detected

Table 4. LoD determination in unpurified NP swabs *in ∑-Virocult (MWE)*

Targets	N1				N2			
RNA Concentration[1]	10^2	10^1	$10^{0.0}$	10^{-1}	10^2	10^1	$10^{0.0}$	10^{-1}
Positives/Total	8/8	8/8	3/8	0/8	8/8	8/8	8/8	2/8
Mean Ct[2]	33,83	36,35	38,01	ND	33,58	36,06	37,61	37,09
Standard deviation (Ct)	0,394	0,769	0,241	-	0,423	0,554	0,299	0,539

1. Concentration is presented in RNA copies/reaction
2. Mean Ct reported for dilutions that are ≥ 95% positive. Calculations only include positive results. ND = not detected

Table 5. LoD determination in unpurified NP swabs in DeltaSwab liquid amies (Deltalab)

Targets	N1				N2			
RNA Concentration[1]	10^2	10^1	$10^{0.0}$	10^{-1}	10^2	10^1	$10^{0.0}$	10^{-1}
Positives/Total	8/8	8/8	6/8	4/8	8/8	8/8	8/8	5/8
Mean Ct[2]	35,22	37,63	37,22	37,65	34,42	35,62	37,34	37,84
Standard deviation (Ct)	0,612	0,972	0,455	0,693	0,591	1,959	0,698	0,576

1. Concentration is presented in RNA copies/reaction
2. Mean Ct reported for dilutions that are ≥ 95% positive. Calculations only include positive results. ND = not detected

Table 6. LoD determination in unpurified NP swabs in *∑-VCM (MWE)*

Targets	N1				N2			
RNA Concentration[1]	10^2	10^1	$10^{0.0}$	10^{-1}	10^2	10^1	$10^{0.0}$	10^{-1}
Positives/Total	8/8	5/8	1/8	0/8	8/8	6/8	2/8	0/8
Mean Ct[2]	35,17	37,22	38,41	ND	34,56	37,29	37,99	ND
Standard deviation (Ct)	0,462	0,816	-	-	0,308	0,763	0,096	-

1. Concentration is presented in RNA copies/reaction
2. Mean Ct reported for dilutions that are ≥ 95% positive. Calculations only include positive results. ND = not detected

Table 7. LoD determination in unpurified NP swabs in ∑-Transwab (MWE)

Targets	N1				N2			
RNA Concentration[1]	10^2	10^1	$10^{0.0}$	10^{-1}	10^2	10^1	$10^{0.0}$	10^{-1}
Positives/Total	8/8	5/8	6/8	0/8	8/8	6/8	6/8	1/8
Mean Ct[2]	35,07	37,79	40,18	ND	34,74	38,97	41,53	ND
Standard deviation (Ct)	0,345	1,419	2,715	-	0,563	3,379	3,874	-

1. Concentration is presented in RNA copies/reaction
2. Mean Ct reported for dilutions that are ≥ 95% positive. Calculations only include positive results. ND = not detected

2. Purified RNAs from validated positive NP swabs were 10-fold serially diluted in DNase/RNase free water and then processed using the kit workflow for purified samples. 2 replicates were realized for each dilution. Quantified SARS-CoV-2 positive control was used to determine viral copy numbers. The LoD is indicated in the yellow case.

Table 8. LoD determination in purified NP swabs

Sample	RNA Concentration[1]	N1		N2		RP	
		Mean Ct[2]	Ct Std. Dev	Mean Ct[2]	Ct Std. Dev	Mean Ct[2]	Ct Std. Dev
9B	10^6	20,84	0,019	20,65	0,167	33,02	0,215
	10^5	24,43	0,102	24,42	0,076	36,84	0,011
	10^4	27,94	0,000	28,00	0,011	38,93	0,000
	10^3	31,66	0,255	31,62	0,034	ND	-
	10^2	34,67	0,035	34,28	0,263	ND	-
	10^1	38,13	0,000	37,75	0,000	0,00	0,000
	10^0	ND	-	ND	-	ND	-
	10^{-1}	ND	-	ND	-	ND	-
9WT	10^3	31,62	0,316	30,81	0,056	32,86	0,136
	10^2	34,85	0,352	34,52	0,227	36,57	0,729
	10^1	36,77	0,098	37,11	0,500	36,88	0,000
	10^0	ND	-	ND	-	ND	-
2A	10^3	30,85	0,146	31,01	0,118	35,42	0,418
	10^2	35,20	0,395	35,00	0,220	39,06	0,459
	10^1	38,04	0,149	37,19	0,112	38,12	0,000
	10^0	ND	-	ND	-	ND	-
12A	10^4	29,10	0,166	29,02	0,138	34,64	0,031
	10^3	32,82	0,093	32,83	0,021	37,09	0,000
	10^2	36,01	0,343	35,94	0,396	ND	-
	10^1	ND	-	ND	-	ND	-
18A	10^3	31,89	0,050	31,77	0,015	33,96	0,091
	10^2	35,50	0,209	35,14	0,479	37,97	1,637
	10^1	ND	-	38,01	0,000	ND	-
	10^0	ND	-	ND	-	ND	-
1B	10^1	37,40	0,000	36,47	0,000	30,97	1,816
	10^0	ND	-	38,05	0,000	33,24	0,105
	10^1	ND	-	ND	-	36,28	0,276
	10^{-2}	ND	-	ND	-	ND	-

1. Concentration is presented in RNA copies/reaction
2. Mean Ct reported for dilutions that are ≥ 95% positive. Calculations only include positive results. ND = not detected

3. Validated positive NP swabs and saliva specimen were 10-fold serially diluted in saline solution, purified with MGISP-NE32 automated extractor, then processed with the kit workflow for purified samples. 8 replicates were realized for each dilution. Quantified SARS-CoV-2 positive control was used to determine viral copy numbers. The LoD is indicated in the yellow case.

Table 9. LoD determination in NP swabs by serially diluted purification

Targets	N1				N2				
RNA Concentration[1]	10^3	10^2	10^1	10^0	10^3	10^2	10^1	10^0	
Positives/Total	8/8	8/8	8/8	2/8	8/8	8/8	8/8	5/8	20A
Mean Ct[2]	29,25	32,26	36,50	37,50	28,75	31,72	35,21	37,42	
Standard deviation (Ct)	0,160	0,214	1,005	0,640	0,209	0,143	0,538	0,472	

Targets	N1				N2				
RNA Concentration[1]	10^3	10^2	10^1	10^0	10^3	10^2	10^1	10^0	
Positives/Total	8/8	8/8	**7/8**	1/8	8/8	8/8	**8/8**	3/8	7B
Mean Ct[2]	29,74	32,90	**35,90**	37,13	28,73	31,89	**34,95**	37,17	
Standard deviation (Ct)	0,097	0,318	**0,322**	-	0,078	0,279	**0,635**	0,412	

1. Concentration is presented in RNA copies/reaction
2. Mean Ct reported for dilutions that are ≥ 95% positive. Calculations only include positive results. ND = not detected
3.

Table 10. LoD determination in saliva specimen by serially diluted purification

Targets	N1					N2					
RNA Concentration[1]	10^4	10^3	10^2	10^1	10^0	10^4	10^3	10^2	10^1	10^0	
Positives/Total	8/8	8/8	8/8	8/8	3/8	8/8	8/8	8/8	8/8	4/8	131/20
Mean Ct[2]	26,76	28,25	32,95	37,05	37,35	25,84	27,58	32,09	35,76	36,70	
Standard deviation (Ct)	0,055	0,045	0,196	0,829	0,616	0,054	0,067	0,152	0,854	0,493	

1. Concentration is presented in RNA copies/reaction
2. Mean Ct reported for dilutions that are ≥ 95% positive. Calculations only include positive results. ND = not detected

Targets	N1					N2					
RNA Concentration[1]	10^4	10^3	10^2	10^1	10^0	10^4	10^3	10^2	10^1	10^0	1
Positives/Total	8/8	8/8	8/8	4/8	0/8	8/8	8/8	8/8	**7/8**	0/8	
Mean Ct[2]	29,92	33,87	36,70	41,04	ND	26,04	29,92	32,85	**35,93**	ND	
Standard deviation (Ct)	0,113	0,223	0,792	1,982	-	0,047	0,057	0,287	**0,732**	-	

Targets	N1					N2					
RNA Concentration[1]	10^4	10^3	10^2	10^1	10^0	10^4	10^3	10^2	10^1	10^0	3
Positives/Total	8/8	8/8	8/8	1/8	0/8	8/8	8/8	8/8	3/8	0/8	
Mean Ct[2]	28,98	32,48	35,71	36,47	ND	28,28	31,64	34,78	36,71	ND	
Standard deviation (Ct)	0,051	0,0224	0,768	-	-	0,065	0,138	0,480	0,083	-	

1. Concentration is presented in RNA copies/reaction
2. Mean Ct reported for dilutions that are ≥ 95% positive. Calculations only include positive results. ND

4. SARS-CoV-2 positive control was 10-fold serially diluted in a pool of NP negative matrices, purified with MGISP-NE32 automated extractor, then processed with the kit workflow for purified samples. 8 replicates were realized for each dilution. Quantified SARS-CoV-2 positive control was used to determine viral copy numbers. The LoD is indicated in the yellow case.

Table 11. LoD determination of SARS-CoV-2 positive control in negative matrix pool by serially diluted purification

Targets	N1				N2				
RNA Concentration[1]	10^3	10^2	10^1	10^0	10^3	10^2	10^1	10^0	SARS-CoV-2 positive control
Positives/Total	8/8	8/8	8/8	0/8	8/8	8/8	8/8	0/8	
Mean Ct[2]	30,82	33,85	36,89	ND	29,97	33,21	36,49	ND	
Standard deviation (Ct)	0,226	0,281	0,795	-	0,174	0,325	0,888	-	

1. Concentration is presented in RNA copies/reaction
2. Mean Ct reported for dilutions that are ≥ 95% positive. Calculations only include positive results. ND = not detected

5. SARS-CoV-2 purified and quantified virus was 10-fold serially diluted in a pool of negative saliva or NP matrices, purified with MGISP-NE32 automated extractor, then processed with the kit workflow for purified samples. 8 replicates were realized for each dilution. The LoD is indicated in the yellow case. (The copy number/reaction was estimated based on 100% extraction efficiency).

Table 12a. LoD determination of SARS-CoV-2 virus in negative saliva pool by serially diluted purification

Targets	N1				N2				
Virus copies/ml *saliva* (copies/reaction)	200.000 (10^3)	20.000 (10^2)	2.000 (10^1)	200 (10^0)	200.000 (10^3)	20.000 (10^2)	2.000 (10^1)	200 (10^0)	SARS-CoV-2 virus purifié
Positives/Total	8/8	8/8	7/8	3/8	8/8	8/8	8/8	3/8	
Mean Ct[1]	30.48	33.88	36.65	38.56	29.55	33.10	36.09	36.56	
Standard Deviation (Ct)	0.148	0.313	0.802	4.711	0.148	0.401	0.691	1.005	

[1] Mean Ct reported for positive values.

Table 12b. LoD determination of SARS-CoV-2 virus in negative NP pool by serially diluted purification

Targets	N1				N2				
Virus copies/ml *saline-NP* (copies/reaction)	200.000 (10^3)	20.000 (10^2)	2.000 (10^1)	200 (10^0)	200.000 (10^3)	20.000 (10^2)	2.000 (10^1)	200 (10^0)	SARS-CoV-2 virus purifié
Positives/Total	8/8	8/8	8/8	3/8	8/8	8/8	8/8	6/8	
Mean Ct[1]	28.57	31.81	35.73	37.57	27.66	30.90	35.19	38.16	
Standard Deviation (Ct)	0.090	0.161	0.350	0.398	0.102	0.125	0.819	1.175	

[1] Mean Ct reported for positive values

In conclusion:

The analytical sensitivity is 2 copies/µL for NP samples.

The analytical sensitivity is 2 copies/µL for saliva samples.

Reactivity (Inclusivity)

In silico analysis of primer and probe sequences

The oligonucleotide primer and probe sequences of the OSANTYS SARS-CoV-2 RT-qPCR Kit were evaluated against 31,623 sequences available in the Global Initiative on Sharing All Influenza Data (GISAID, https://www.gisaid.org) database as of June 20, 2020, to demonstrate the predicted inclusivity of … Diagnostic Panel. Nucleotide mismatches in the primer/probe regions with frequencies > 0.1% are shown below. With the exception of one nucleotide mismatch with frequency > 1% (2.00%) at the third position of the N1 probe, the frequency of all mismatches was < 1%, indicating that prevalence of the mismatches were sporadic. Only one sequence (0.0032%) had two nucleotide mismatches in the N1 probe, and one other sequence from a different isolate (0.0032%) had two nucleotide mismatches in the N1 reverse primer. No sequences were found to have more than one mismatch in any N2 primer/probe region. The risk of these mismatches resulting in a significant loss in reactivity causing a false negative result is

extremely low due to the design of the primers and probes, with melting temperatures > 65°C and with annealing temperature at 63.1°C that can tolerate up to two mismatches.

Table 13. In Silico Inclusivity Analysis of OSANTYS SARS-CoV-2 RT-qPCR Panel among 31,623 genome sequences available from GISAID as of June 20, 2020

Primer/Probe	N1 probe	N1 reverse		N2 probe
Location (5'>3')	3	15	21	13
Mismatch Nucleotide	C>T	G>T	T>C	C>T
Mismatch No.	632	34	71	46
Mismatch Frequency (%)	2.00	0.11	0.22	0.15

In silico and in vitro analysis of SARS-CoV-2 variants

Sequence analysis of the N gene from Alpha, V1/S.501Y.V1 , Beta, V2/S.501Y.V2 , Gamma, V3/S.501Y.V3, Delta, 21A, 21I, 21J, Epsilon, 21C, Kappa, 21B variants revealed that N1 and N2 primer/probe sets are not in the regions affected by mutations, thus they can be detected by this assay, excluding any false negative result.

Sequence analysis of the N gene from the Omicron variants (BA.1, BA.2, BA.4, BA.5, BA.2.12.1) revealed that the N2 set of primers and probe is not in the regions affected by mutations. The mutation present on the 3rd base in 5' of the N1 probe does not impact the detection of the N1 target, as shown by Yanxia Bei *et al*. (5). Thus, the Omicron variants can be detected by this assay, excluding any false negative result.

In addition to the *in silico* analysis, RNAs purified from validated Alpha, Beta, Gamma, Delta and Omicron variants were tested with this assay together with the wild-type strand (WT). The data demonstrate that the expected results were obtained.

Table 14. Detection of SARS CoV-2 variants

Targets	N1					N2				
Variant	WT	Alpha	Beta & Gamma	Delta	Omicron	WT	Alpha	Beta & Gamma	Delta	Omicron
Positives/Total	10/10	20/20	10/10	19/19	15/15	10/10	20/20	10/10	19/19	15/15

Specificity/Exclusivity testing: in silico analysis

BLASTn analysis queries of the SARS-CoV-2 assay primers and probes were performed against public domain nucleotide sequences. The database search parameters were as follows:
1) The nucleotide collection consists of GenBank+EMBL+DDBJ+PDB+RefSeq sequences, but excludes EST, STS, GSS, WGS, TSA, patent sequences as well as phase 0, 1, and 2 HTGS sequences and sequences longer than 100Mb.
2) The database is non-redundant. Identical sequences have been merged into one entry, while preserving the accession, GI, title and taxonomy information for each entry.
3) Database was updated on 10/03/2019.
4) The search parameters automatically adjust for short input sequences and the expect threshold is 1000.
5) The match and mismatch scores are 1 and -3, respectively.
6) The penalty to create and extend a gap in an alignment is 5 and 2 respectively.

SARS-CoV-2 N1 Assay

N1 Probe sequence showed high sequence homology with SARS coronavirus and Bat SARS-like coronavirus genome. However, N1 forward and reverse primers showed no sequence homology with SARS coronavirus and Bat SARS-like coronavirus genome. Combining primers and probe, there is no significant homologies with human genome, other coronaviruses or human microflora that would predict potential false positive RT-qPCR results.

SARS-CoV-2 N2 Assay

The N2 forward primer sequence showed high sequence homology to Bat SARS-like coronaviruses. The N2 reverse primer and N2 probe sequences showed no significant homology with human genome, other coronaviruses or human microflora. Combining primers and probe, there is no prediction of potential false positive RT-qPCR results.

In summary, the N1 and N2 primer/probe sets designed for the specific detection of SARS-CoV-2, showed no significant combined homologies with human genome, other coronaviruses, or human microflora that would predict potential false positive RT-qPCR results.

In addition to the *in silico* analysis, several organisms were extracted and tested in triplicates with the OSANTYS SARS-CoV-2 RT-qPCR Kit to demonstrate analytical specificity and exclusivity. Studies were performed with nucleic acids extracted using the MGISP-NE32 automated extraction system. The data demonstrate that expected results are obtained.

Table 15. Specificity/Exclusivity of the OSANTYS SARS-CoV-2 RT-qPCR Kit

Pathogen	Source	N1	N2	RP
Human coronavirus 229E	Viral culture	ND	ND	25.31
Human coronavirus OC43	Viral culture	ND	ND	24.90
Human coronavirus NL63	Viral culture	ND	ND	33.96
MERS-coronavirus	Purified virus*	ND	ND	31.59
SARS-coronavirus	Purified virus*	ND	ND	29.82
Influenza A (H1N1)	Purified virus*	ND	ND	31.58

*genomic matrix was added to mimic the biological sample

Endogenous interference substances studies

To evaluate interfering substances in unpurified and purified samples, pooled SARS-CoV-2 negative NP swabs were spiked with SARS-CoV-2 positive control at 2.000 copies/µl, pooled SARS-CoV-2 negative saliva specimen were spiked with SARS-CoV-2 purified and quantified virus at 100.000 copies/ml, and potential interfering substances at the concentration reported in Table 16 (a/b). Each substance was tested in triplicate, (except the blood tested in duplicate for NP swabs), following or not nucleic acid extraction. No false negative results were observed for any of the substances at the concentrations tested as illustrated in Tables 16a and 16b.

Pooled SARS-CoV-2 negative NP swabs and saliva specimen were spiked with potential interfering substances at the concentration reported in Table 16 (a/b). Each substance was tested in triplicate, (except the blood tested in duplicate for NP swabs), following or not nucleic acid extraction. The results are showed in Table XX. No false positive results were observed for any of the substances at the concentrations tested as illustrated in Tables 16a and 16b.

Table 16a. Analysis of endogenous interference substances in NP swab specimen

Interfering substance	Final concentration in sample	Agreement with expected results			
		Unpurified positive NP samples	Purified positive NP samples	Unpurified negative NP samples	Purified negative NP samples
None	N/A	3/3	3/3	3/3	3/3
Blood (human)	1% v/v	2/2	2/2	2/2	2/2
Nasal corticosteroids—Dymista™	5 µg/mL	3/3	3/3	3/3	3/3
Throat lozenges, oral anesthetic and analgesic—Xylocaine 5%	1% w/v	3/3	3/3	3/3	3/3
Oseltamivir phosphate - Tamiflu	33 µg/mL	3/3	3/3	3/3	3/3
Antibiotic, nasal ointment—Mupirocin 2%	5 µg/mL	3/3	3/3	3/3	3/3
Antibacterial, systemic—Tobramycin - Nebcine	0.6 mg/mL	3/3	3/3	3/3	3/3
Physiological Serum 0.9%	10% v/v	3/3	3/3	3/3	3/3

Table 16b. Analysis of endogenous interference substances in saliva specimen

Interfering substance	Final concentration in sample	Agreement with expected results	
		Purified positive saliva samples	Purified negative saliva samples
None	N/A	3/3	3/3
Blood (human)	1% v/v	3/3	3/3
Nasal corticosteroids—Dymista™	5 µg/mL	3/3	3/3
Throat lozenges, oral anesthetic and analgesic—Xylocaine 5%	1% w/v	3/3	3/3
Oseltamivir phosphate - Tamiflu	33 µg/mL	3/3	3/3
Antibiotic, nasal ointment—Mupirocin 2%	5 µg/mL	3/3	3/3
Antibacterial, systemic—Tobramycin - Nebcine	0.6 mg/mL	3/3	3/3
Physiological Serum 0.9%	10% v/v	3/3	3/3

Clinical performance

A clinical evaluation study was performed to evaluate the performance of the OSANTYS SARS-CoV-2 RT-qPCR Kit using NP swabs and saliva specimen previously tested positive or negative, following or not RNA extraction.

A total of *169 positive specimen* were tested:

- *32 positive NP swab specimen from purified RNA method* and Bosphore SARS-CoV-2/Flu/RSV Panel Kit (Anatolia geneworks)

- *97 positive NP swab specimen from direct PCR* (Novel Coronavirus (2019-nCoV) Nucleic Acid Diagnostic Kit (PCR-Fluorescence Probing) – Sansure Biotech and Detection Expert SARS-CoV-2 – Genestore)
- *40 positive saliva specimen from purified RNA method* and Bosphore SARS-CoV-2/Flu/RSV Panel Kit (Anatolia geneworks)

In addition to the positive specimen, *168 negative specimen* were tested:

- *10 negative NP swab specimen from purified RNA method* and Bosphore SARS-CoV-2/Flu/RSV Panel Kit (Anatolia geneworks)
- *134 negative NP swab specimen from direct PCR* (Novel Coronavirus (2019-nCoV) Nucleic Acid Diagnostic Kit (PCR-Fluorescence Probing) – Sansure Biotech and Detection Expert SARS-CoV-2 – Genestore)
- *24 negative saliva specimen salivaires from purified RNA method* and Bosphore SARS-CoV-2/Flu/RSV Panel Kit (Anatolia geneworks) or *from direct PCR* (Novel Coronavirus (2019-nCoV) Nucleic Acid Diagnostic Kit (PCR-Fluorescence Probing) – Sansure Biotech

Table 17. NP swab clinical evaluation study on purified RNA

Results	MGISP-NE32
Positive/Total	32/32
Negative/Total	10/10
Inconclusive/Total	0/42
Invalid/Total	0/42

The clinical sensitivity of the OSANTYS SARS-CoV-2 RT-qPCR KIT on purified NP sample is 100%.

The clinical specificity of the OSANTYS SARS-CoV-2 RT-qPCR KIT on purified NP sample is 100%.

Table 18. NP swab clinical evaluation study on unpurified samples

Results	DIRECT RT-qPCR
Positive/Total	94*/97
Negative/Total	133**/134
Inconclusive/Total	0/231
Invalid/Total	0/231

***** Reference method N2 Ct > 34 for 3 samples. 6 or more days storage at -20°C for non-detected samples

** N1 Ct = 37,93 and N2 Ct = 36,30 for the positive detected sample confirmed by purified RNA

The clinical sensitivity of the OSANTYS SARS-CoV-2 RT-qPCR KIT on unpurified NP sample is 96.9%.

The clinical specificity of the OSANTYS SARS-CoV-2 RT-qPCR KIT on unpurified NP sample is 99.2%.

Table 19. Saliva clinical evaluation study on purified RNA

Results	MGISP-NE32
Positive/Total	40/40
Negative/Total	23*/24
Inconclusive/Total	0/64
Invalid/Total	0/64

*Ct N1 = 40,37 and Ct N2 = 35 for the positive detected sample (Reference method: direct RT-qPCR)

The clinical sensitivity of the OSANTYS SARS-CoV-2 RT-qPCR KIT test on saliva samples is 100%. The 95% confidence interval is (0.91 - 1.0).

References

1. Ballew, H. C., *et al*. “Basic Laboratory Methods in Virology,” DHHS, Public Health Service 1975 (Revised 1981), Centers for Disease Control and Prevention, Atlanta, Georgia 30333.

2. Clinical Laboratory Standards Institute (CLSI), “Collection, Transport, Preparation and Storage of Specimens for Molecular Methods: Proposed Guideline,” MM13-A

3. Lieber, M., *et al*. "A Continuous Tumor Cell Line from a Human Lung Carcinoma with Properties of Type II Alveolar Epithelial Cells." *International Journal of Cancer* 1976, 17(1), 62-70.

4. CDC 2019-Novel Coronavirus (2019-nCoV) Real-Time RT-PCR Diagnostic Panel. CDC-006-00019, Revision: 05.

5. Yanxia Bei, *et al*., The Omicron variant mutation at position 28,311 in the SARS-CoV-2 N gene does not perturb CDC N1 target detection. medRxiv 2021.12.16.21267734

Contact Information, Ordering, Customer and Product Support

For additional documentation and information about this kit, visit: www.osantys.com
For technical and product support, send an email to: contact@osantys.com
For orders, send an email to: orders@osantys.com

Protocol At-A-Glance

1. Thaw reagents at room temperature
2. Mix and spin the reagent tubes briefly before use
3. Set up reaction plate or tubes as follows:

Component	Volume/Reaction
One-Step RT-qPCR Enzyme Master Mix 2X	N x 12.5 µL
SARS-CoV-2 Primer/Probe Mix	N x 7.5 µL
Total Volume	**N x 20.0 µL**

4. Add 5 µL of each sample (purified RNA or unpurified sample), 5 µL of negative control and 5 µL of positive controls.
5. Spin briefly
6. Create the following thermal protocol:
   - Step 1: 52°, 5 min
   - Step 2: 95°, 10 sec
   - Step 3: 95°C, 5 sec
     63.1°C, 30 sec (+ plate read) } 45 cycles
7. Select reporter dyes:

Target	Reporter Dye
N1	FAM/blue
N2	VIC/green
RP	Cy5/red

8. Indicate 25 µl as reaction volume per well.
9. Load the strip tubes or plate and start the run.
10. After the instrument run is complete, analyze the data following instrument manual.