PhoenixDx® ***SARS-CoV-2 Multiplex Plus***

for diagnostic use

qualitative RT-PCR-based detection of SARS-CoV-2 ***PLUS*** *Delta and Omicron variant identification*

INSTRUCTIONS FOR USE

96 Tests // 960 Tests

REF **PCCSKU15297 // PCCSKU15298**

v 1.0

CE IVD

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1) Intended Use

PHOENIXDX® SARS-COV-2 MULTIPLEX PLUS is a real-time RT-PCR-based diagnostic test for the *in vitro* qualitative detection and discrimination of SARS-CoV-2 and the P681R mutant in respiratory specimens and sera from patients who meet COVID-19 clinical and/or epidemiological criteria.

PHOENIXDX® SARS-COV-2 MULTIPLEX PLUS detects SARS-CoV-2 RNA in nasopharyngeal and oropharyngeal swab samples during infection. Positive results indicate the presence of SARS-CoV-2 RNA; clinical correlation with patient history and other diagnostic information must be considered to determine the actual patient infection status. Positive results do not exclude bacterial infection or co-infection with other viruses.

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

The use of **PHOENIXDX® SARS-COV-2 MULTIPLEX PLUS** is intended for use by clinical laboratory personnel specifically instructed and trained in the techniques of real-time PCR and *in vitro* diagnostic procedures.

2) PhoenixDx® Detection System

PHOENIXDX® SARS-COV-2 MULTIPLEX PLUS is a real-time RT-PCR-based detection and discrimination system for SARS-CoV-2. SARS-CoV-2 is considered a novel human coronavirus that is genetically distinct from the common human coronaviruses (229E, NL63, OC43, HKU1), which cause seasonal acute respiratory illness. It is also genetically distinct from the two newer human coronaviruses, MERS-CoV and SARS-CoV.

PHOENIXDX® SARS-COV-2 MULTIPLEX PLUS detects a target sequence highly specific for SARS-CoV-2 (N gene), mutant P681R SARS-CoV-2 (S gene), mutant E484A SARS-CoV-2 (S Gene) and one sequence specific for human RNA (RNAse P) serving as a human extraction control (**HEC**). Additionally, three non-infectious target positive controls (**TPC**) are included. The positive controls are used to confirm functionality of the assays, overall PCR performance and assist with data interpretation of patient samples. The human extraction control is used to evaluate the quality of the RNA isolation independently from the SARS-CoV-2 assays in a different detection channel.

2.1) qPCR-based Detection

The first step in the detection of SARS-CoV-2 and the discrimination of mutant E484A SARS CoV-2 and mutant P681R SARS-CoV-2 is the conversion of viral RNA into cDNA. Afterwards, the target sequences and the **HEC** are simultaneously amplified in one reaction with amplification monitored in real time through the use of fluorescently labelled probes: upon incorporation into the newly amplified DNA strands, the fluorophore is released and an increase in fluorescence signal can be observed.

Due to the intrinsic mutation rate of coronaviruses, it is possible that mutations in the target sequence occur and accumulate over time. This can lead to false-negative results with a PCR-based detection approach.

Samples tested positive should always be confirmed through complementary methods and additional analysis in an independent laboratory.

PHOENIXDX® SARS-COV-2 MULTIPLEX PLUS is compatible with every qPCR cycler with calibrated Cy5, ROX, HEX/VIC and FAM™.

2.2) Materials Provided

Quantity & Volume (96 Tests)	Quantity & Volume (960 Tests)	Component
1x 100 µl	1x 1 ml	20X RT Enzyme Mix
1x 400 µl	5x 800 µl	5X MTM Buffer
1x 100 µl	1x 1 ml	Multiplex Plus Assay Mix
1x 100 µl	1x 100 µl	SC2 Delta TPC
1x 100 µl	1x 100 µl	SC2 Omicron TPC
1x 100 µl	1x 100 µl	SC2 Wildtype TPC

2.3) Additional Materials Required

- Suitable means & equipment for nucleic acid extraction (see chapter 3.4)
- Real-time PCR detection system equipped for Cy5, ROX, HEX/VIC and FAM™ detection
- Adjustable pipettes & fitting filtered pipette tips
- Nuclease-free water
- Appropriate PSA & workspaces for working with potentially infectious samples
- Surface decontaminants such as DNA-Excitus (Applichem), 10% bleach (1:10 dilution of commercial 5.25-6.0% sodium hypochlorite)
- Nuclease-free tubes / strips / plates to prepare dilutions, mastermixes etc.
- Nuclease-free tubes / strips / plates corresponding to the PCR device
- Suitable storage options for reagents and specimen (4°C, -20°C, -70°C)

2.4) Storage

- Store all components at -20°C and avoid repeated freeze and thaw cycles (≤ 3 freeze/thaw cycles; prepare aliquots if required).
- Protect the **Multiplex Plus Assay Mix** from light as prolonged exposure can diminish the performance of the fluorophores.
- If the kit components have been damaged during transport, contact Procomcure Biotech. Do not use as performance may be compromised.
- Keep reagents separate from sample material to avoid contamination.
- Do not use after the designated expiry date.

3) Considerations before Starting

3.1) Biosafety

- Wear appropriate personal protective equipment (e.g., gowns, powder-free gloves, eye protection) when working with clinical specimens.
- Specimen processing should be performed in a certified class II biological safety cabinet following biosafety level 2 or higher guidelines.
- For more information, refer to:
    - Interim Guidelines for Collecting, Handling, and Testing Clinical Specimens from Patients Under Investigation (PUIs) for 2019 Novel Coronavirus (SARS-COV-2) https://www.cdc.gov/coronavirus/2019-nCoV/guidelines-clinical-specimens.html
    - Biosafety in Microbiological and Biomedical Laboratories 5th edition available at http://www.cdc.gov/biosafety/publications/.
- The use of **PHOENIXDX® SARS-COV-2 MULTIPLEX PLUS** and data evaluation is restricted to trained laboratory personnel only.
- Good laboratory practice is essential for optimal performance of this assay. Special care must be taken avoid contamination of the components of the kit. All reagents must be closely monitored for impurities and contamination. Discard suspicious reagents according to local guidelines and regulations.

3.2) Specimens

Only use appropriate specimens for testing, such as:

- Respiratory specimens including nasopharyngeal / oropharyngeal aspirates or washes, nasopharyngeal / oropharyngeal swabs, broncheoalveolar lavage, tracheal aspirates and sputum.
- Swab specimens should be collected only on swabs with a synthetic tip (such as polyester or Dacron®) with aluminum or plastic shafts. Swabs with calcium alginate or cotton tips with wooden shafts are not recommended as they may contain substances that inactivate some viruses and inhibit PCR testing and should only be used if dacron or rayon swabs are not available.
- Saliva samples collected with PhoenixDx® Gargling/Saliva Collection Kit

3.3) Specimens - Handling and Storage

- Specimens can be stored at 4°C for up to 72 hours after collection.
- If a delay in extraction is expected, store specimens at -70°C or lower.
- Clinical specimens must be considered potentially infectious and treated accordingly.

⚠ Do not vortex specimens as this will fragment the RNA and lead to failure of **PHOENIXDX® SARS-COV-2 MULTIPLEX PLUS.**

Do not use specimens if

- they were not kept at 2-4°C (≤ 4 days) or frozen at -70°C or below (4 ≤ 120 days).
- they are insufficiently labelled or lack documentation.
- they are not suitable for this purpose (see above for suitable sample material).
- the specimen volume is insufficient.

3.4) Sample Preparation / Nucleic Acid Extraction

- The performance of RT-PCR assays strongly depends on the amount and quality of sample template RNA. It is strongly recommended to qualify and validate RNA extraction procedures for recovery and purity before testing specimens.
- Suitable nucleic acid extraction systems successfully used in combination with **PhoenixDx® Detection Kits** include: **SphaeraMag® DNA/RNA Isolation Kits**, **Phoenix Virus NAT®**, Quick-RNA Viral Kits (Zymo Research), bioMérieux NucliSens® systems, QIAamp® Viral RNA Mini Kit, QIAamp® MinElute Virus Spin Kit or RNeasy® Mini Kit (QIAGEN), EZ1 DSP Virus Kit (QIAGEN), Roche MagNA Pure Compact RNA Isolation Kit, Roche MagNA Pure Compact Nucleic Acid Isolation Kit.
- Only extract the number of specimens that will be tested in a single day.
- Do not freeze/thaw extracts more than once before testing as each freeze/thaw cycle will decrease the RNA quality. For optimal results, use directly and do not freeze and thaw before use.
- Extracted nucleic acids should be stored at -70°C or lower and (if re-testing is expected) stored in aliquots.

3.5) Reaction Setup

1) Make sure that all necessary equipment and devices are suitable, calibrated and functional before starting the experiments.
2) Decontaminate equipment and workspace and prepare everything needed for the following experiment to keep the workflow short and repeatable.
3) Switch on the PCR detection system and program it to avoid delays after setting up the reactions.
4) Thaw all components of **PHOENIXDX® SARS-COV-2 MULTIPLEX PLUS** on ice and mix gently but thoroughly to ensure even distribution of components. Collect liquid at the bottom of the tube with a quick spin.
5) Set up your **Mastermix Plate**:

    a. Always prepare negative control reactions with nuclease-free dH_2O instead of sample material **(NTC)** to detect contamination in your reagents.
    b. When using the provided target positive control **(TPC)**, use **14 µl / reaction**.
    c. > 2 replicates / sample are strongly recommended.
    d. Prepare enough mastermix for all planned reactions. It is recommended to prepare mastermix for 2 additional reactions to compensate for pipetting inaccuracies.
    e. Distribute the mastermix to your strips/plate.

Component	Volume
20X RT Enzyme Mix	1 µl
5X MTM Buffer	4 µl
Multiplex Plus Assay Mix	1 µl
Isolated sample RNA / TPCs / NTC	14 µl / 14 µl / 14µl dH_2O

6) Transfer the Mastermix Plate to a separate workspace to add the sample material. Preparing reagents and final reaction setup in separate workspaces helps to avoid contamination of equipment and reagents with sample material.

   a. Prepare negative reactions first and seal them before handling positive samples. It is recommended to only bring potentially positive sample material and the included target positive control to the workspace once the NTC is prepared and sealed.
   b. Add your samples to the Mastermix Plate.
   c. Keep reactions on ice until transferring them to the PCR device.

7) Transfer the reactions to the PCR device, then cycle according to these guidelines:

Step	Cycles	Temperature	Duration
Reverse Transcription	1	50°C	5 minutes
Initial Denaturation	1	95°C	5 minutes
Amplification	40	95°C	5 seconds
		59°C[1]	45 seconds

[1] Enable Data Collection for **FAM™** (P681R – Delta variant), **HEX/VIC** (E484A – Omicron variant), **ROX** (SARS-CoV-2 // N Gene) and **Cy5** (HEC // RNAse P). **Do not set ROX as passive reference since the channel is used for detection!**

Once the run is finished, do not open the reaction tubes to avoid contamination and discard according to local guidelines and regulations. Do not autoclave as this may contaminate laboratory equipment with amplicons.

4) Analysis

Any signal recorded in the FAM™ or HEX/VIC channels may only be analyzed if the corresponding SARS-CoV-2 Ct value in the ROX channel is ≤ 30. Sole detection of SARS-CoV-2, however, may still be performed with a Ct value in the ROX channel ≤ 35.

- **dH_2O controls (NTC) must not give a Ct value for any assay**. If they do, the reaction was contaminated with sample RNA / cDNA. Decontaminate equipment and workspace and repeat the reactions. Also, check for device-derived artifacts or falsely placed threshold. **If a contamination persists, use fresh reagents**.

- **For a sample to be considered positive for SARS-CoV-2, the ROX channel must give a positive Ct value.** Amplification of the HEC in the **Cy5 channel** is expected around Ct 22-32. Should the **HEC** fail to amplify, the sample must still be considered positive. This outcome is possible when having an unusually high virus titer, or the sample was not of human origin, but cell culture derived or after analysis for surface contamination.

- **For a sample to be considered positive for P681R (Delta) SARS-CoV-2, the FAM™ channel must give a positive Ct value and the HEX/VIC channel must give no or delayed Ct value with significantly lower amplification curve compared to the FAM™ channel.** Amplification of the HEC in the **Cy5 channel** is expected around Ct 22-32. Should the **HEC** fail to amplify, the sample must still be considered positive. This outcome is possible when having an unusually high virus titer, or the sample was not of human origin, but cell culture derived or analysis of surface contamination.

- **For a sample to be considered positive for E484A (Omicron) SARS-CoV-2, the HEX/VIC channel must give a positive Ct value and the FAM™ channel must give no or delayed Ct value with significantly lower amplification curve compared to the HEX/VIC channel.** Amplification of the HEC in the **Cy5 channel** is expected around Ct 22-32. Should the **HEC** fail to amplify, the sample must still be considered positive. This outcome is possible when having an unusually high virus titer, or the sample was not of human origin, but cell culture derived or analysis of surface contamination.

- **For a sample to be considered positive for other variants without E484A and P681R mutated SNPs, neither the HEX/VIC channel nor the FAM™ channel must give positive Ct values.** Amplification of the HEC in the **Cy5 channel** is expected around Ct 22-32. Should the **HEC** fail to amplify, the sample must still be considered positive. This outcome is possible when having an unusually high virus titer, or the sample was not of human origin, but cell culture derived or analysis of surface contamination.

- **For a sample to be considered negative for SARS-CoV-2, the FAM, HEX/VIC and ROX channels must not give a positive Ct value,** but the amplification of the HEC in the **Cy5 channel** is expected around Ct 22-32.

- **If amplification in the FAM™ channel or HEX/VIC channel in combination with the Cy5 channel is observed but not in the ROX channel, the SARS-CoV-2 assay has failed.** This can indicate a new mutation in the target sequence or a flawed reaction setup. Repeat the reaction and if the result persists, employ complementary methods to check for potential mutations.

- **If no amplification signal in neither the FAM™ or HEC/VIC channels nor the ROX or Cy5 channels are observed, PCR was inhibited.** Check reaction setup and device settings and repeat the RNA extraction if necessary. Results are invalid and cannot be interpreted.

- **All reactions containing RNA isolate must give positive Ct values for the HEC assay when working with samples of human origin. The Ct values are expected around 22-32.** Failure to amplify the negative human extraction control indicates a flawed RNA extraction or loss of RNA isolate due to RNAse contamination. Late Ct values for the **HEC** may indicate a low RNA quality / amount in the extract.

⚠ **Always analyze your sample reactions independently of the TPC reactions**. The TPC is an artificial control construct resulting in a significantly higher signal strength than actual samples. This will lead to a distorted picture when analyzed together with actual samples.
For analysis, the **threshold must be set only for the wells containing sample material** not including wells with TPC reactions. It is recommended to set each target threshold manually for best results. If amplification in sample reactions seems to have failed, check if the TPC reactions are displayed simultaneously.

Table 1 Interpretation of amplification results with PHOENIXDX® SARS-COV-2 MULTIPLEX PLUS

FAM™	HEX/VIC	ROX	Cy5	Interpretation
+	/	+	+	P681R SARS-CoV-2, SARS-CoV-2 target sequences and HEC were amplified. The **sample is considered positive for Delta SARS-CoV-2**. **Expected result for SC2 Delta TPC.**
/	+	+	+	E484A SARS-CoV-2, SARS-CoV-2 target sequence and HEC were amplified. The **sample is considered positive for mutant P681R SARS-CoV-2**. **Expected result for SC2 Omicron TPC.**
/	/	+	+	Neither E484A nor P681R SARS-CoV-2 were detected, but the conserved SARS-CoV-2 target and the HEC were amplified. The **sample is considered positive for SARS-CoV-2**, possibly with E484Q, E484K, P681H, 681P or 484E SNPs, but also other spontaneous variants around the S:681 and S:484 loci cannot be excluded. **Expected result for SC2 Wiltype TPC.**
/	/	/	+	Only the target sequence for the **HEC** was amplified. **The sample is considered negative for SARS-CoV-2**.
≥ 1 channel gives positive Ct value		/	+	P681R and/or E484A SARS-CoV-2 target sequences were detected but not SARS-CoV-2 target sequence. This can indicate a new mutation in the N gene or flawed reaction setup. Repeat the reaction.

FAM™	HEX/VIC	ROX	Cy5	Interpretation
≥ 1 channel gives positive Ct value		+	/	**SARS-CoV-2** target sequences were amplified (N gene and/or S gene) but not the **HEC**. **The sample must still be considered positive for SARS-CoV-2 and/or P681R/E484A SARS-CoV-2**. This outcome is possible when having an unusually high virus titer, or the sample was not of human origin, but cell culture derived or analysis of surface contamination.
/	/	/	/	No viral or human RNA detected. RNA might have been degraded or PCR was inhibited. Results are invalid for data interpretation. **Expected result for the NTC.**

Table 2 Interpretation of controls provided with PHOENIXDX® SARS-COV-2 MULTIPLEX PLUS

5) Limitations

- For reliable results, it is essential to adhere to the guidelines given in this manual. Changes in reaction setup or cycling protocol may lead to failed experiments.
- Depending on the sample matrix, inhibitors may be present in the isolated RNA and disable reverse transcription and / or PCR amplification. If this is the case, another sample type or isolation method may be beneficial.
- Spontaneous mutations within the target sequence may result in failure to detect the target sequence.
- Any signal recorded in the FAM™ and HEX/VIC channels may only be analyzed if the corresponding SARS-CoV-2 Ct value in the ROX channel is ≤ 30. Sole detection of SARS-CoV-2, however, may still be performed with a Ct value in the ROX channel ≤ 35.
- For safety reasons, specimen collection, transport, storage and processing procedures must be performed by trained personnel only.
- This assay must not be used on specimens directly. Appropriate nucleic acid extraction methods have to be conducted prior to using this assay.
- Reliable results depend strongly on proper sample collection, storage and handling procedures.

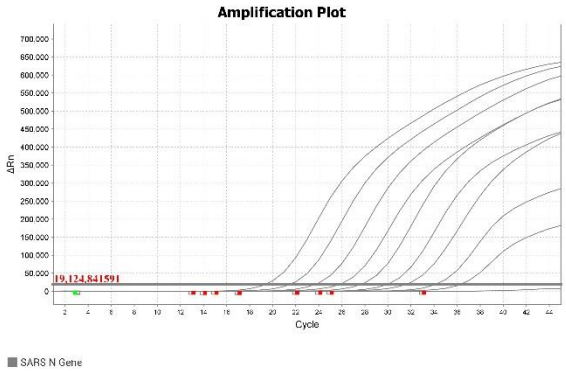
6) Quality Control

In accordance with Procomcure Biotech GmbH's EN ISO 13485-certified Quality Management System, each lot of **PHOENIXDX® SARS-COV-2 MULTIPLEX PLUS** is tested against predetermined specifications to ensure consistent product quality.

7) Analytical Sensitivity & Linearity

The LOD95(Limit of Detection) defines the number of target sequences (copy number) that can be detected in ≥ 95% of reactions. The LOD95 was determined by testing a serial dilution of isolated SARS-CoV-2 RNA with 9 concentrations in 24 replicates per concentration in a 1:4 serial dilution manner. The LOD95 for SARS-CoV-2 was determined to be 3.4 copies / µl RNA.

ROX Channel (conserved N gene)



8) CLINICAL DATA

The performance of **PHOENIXDX® SARS-COV-2 MULTIPLEX PLUS** was tested in a paired comparison using collected nasopharyngeal swabs. **PHOENIXDX® SARS-COV-2 MULTIPLEX PLUS** was evaluated using clinical samples (36 samples for P681R SARS-CoV-2 and 34 samples of E484A SARS-CoV-2) collected from patients with signs and symptoms of an upper respiratory infection against next-generation genome sequencing Illumina COVIDSeq Test (#20044461) MiSeq® Reagent Kit v3 (MS-102-3001). RNA isolation was automatically performed using the **SphaeraMag® DNA/RNA Isolation Kit**, a magnetic-bead-based isolation kit according to the instructions provided by the manufacturer. Clinical samples were collected by qualified personnel according to the instructions provided by the manufacturer of the collection device. Samples were tested to be negative with a commercially available nucleic acid test for the qualitative detection of microorganisms associated with common upper respiratory tract infections.

Reference Method	n	PHOENIXDX® SARS-COV-2 MULTIPLEX PLUS	
		positive	negative
Positive	70	A= 69	B= 1
Negative	30	C= 0	D= 30
Clinical sensitivity			98.5 %
Clinical specificity			100 %

Reference Method	n	PHOENIXDX® SARS-COV-2 MULTIPLEX PLUS		
		P681R (FAM)	E484A (HEX/VIC)	(no FAM, no HEX/VIC; but ROX and Cy5)
P681R SNP	36	35	0	1
E484A SNP	34	0	32	2
Conformity rate		97.2 %	94.1 %	

Validation of PhoenixDx® Gargling/Saliva Collection Kit for Sampling

PhoenixDx® Gargling/Saliva Collection Kit was validated for use in SARS-CoV-2 PCR diagnostic tests in a comparative approach with 48 SARS-CoV-2 positive patients. Each patient was sampled with a pharyngeal swab, a nasopharyngeal swab and the gargling set. RNA Isolation was performed automatically with the SphaeraMag® DNA/RNA isolation system, eluates were tested for presence of SARS-CoV-2 RNA and the human extraction control (**HEC**) using the PhoenixDx® SARS-CoV-2 Multiplex Kit. To evaluate the performance of all three sampling methods, CT values for the **HEC** were compared:

Sampling Method	Ct values for HEC	Ct values for SARS-CoV-2
Pharyngeal Swab	28.455 ± 1.861	38.545 ± 3.241
Nasopharyngeal Swab	25.333 ± 2.182	31.462 ± 5.102
Gargling Set	24.067 ± 4.307	33.267 ± 3.397

While the mean variation of the **HEC** Ct values is highest for the gargling set, the median Ct value is lowest for both viral and human target when using the gargling set followed by the nasopharyngeal swab and the pharyngeal swab. Therefore, the PhoenixDx® Gargling/Saliva Collection Kit is a valid sampling method for SARS-CoV-2 diagnostic.

9) Trademarks

PhoenixDx®, SphaeraMag®, NucliSens® (bioMérieux), QIAamp®, RNeasy® (QIAGEN), ChargeSwitch® (Invitrogen), ROX™, FAM™ (Life Technologies), DNAZap™, DNA Away™, RNAse Away™

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10) Literature

Corman VM, Landt O, Kaiser M, et al. Detection of 2019 novel coronavirus (2019-nCoV) by real-time RT-PCR. Euro Surveill. 2020;25(3):2000045. doi:10.2807/1560-7917.ES.2020.25.3.2000045

11) Technical Assistance

For questions or technical support, contact Procomcure Biotech:

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12) Symbol Definition (Manual & Packaging)

	Contains sufficient for <n> tests
	Catalogue Number
	Manufacturer
	Batch Code
	Temperature Limit
	Use-by Date
	Consult instructions for use
	In vitro diagnostic

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