PHOENIXDX® *SARS-COV-2 MUTANT SCREEN [DEL HV69/70]*

for laboratory use

qualitative RT-PCR-based kit for the discrimination of wildtype SARS-CoV-2 and del HV69/70 mutant SARS-CoV-2

INSTRUCTIONS FOR USE

96 Tests

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v 1.0

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1) Intended Use

PhoenixDx® SARS-CoV-2 Mutant Screen [del HV69/70] is a real-time RT-PCR-based test system for the *in vitro* discrimination between wildtype SARS-CoV-2 and del HV69/70 mutant SARS-CoV-2 in respiratory specimens and sera. The kit is intended to detect the del HV69/70 mutation in samples already tested positive for SARS-CoV-2.

PhoenixDx® SARS-CoV-2 Mutant Screen [del HV69/70] detects wildtype SARS-CoV-2 and delHV69/70 mutant SARS-CoV-2 RNA in nasopharyngeal and oropharyngeal swab samples during infection. Positive results indicate the presence of wildtype SARS-CoV-2 and delHV69/70 mutant SARS-CoV-2, further analysis shows if the wildtype or mutant virus variant is present; 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 an infection with SARS-CoV-2 (wildtype or mutant) 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 Mutant Screen [del HV69/70]** is intended for use by clinical laboratory personnel specifically instructed and trained in the techniques of real-time PCR.

2) PhoenixDx® Detection System

PhoenixDx® SARS- CoV-2 Mutant Screen [del HV69/70] is a real-time RT-PCR-based test system for the *in vitro* discrimination between wildtype SARS-CoV-2 and del HV69/70 mutant SARS-CoV-2.

Table 1 Reported mutants of SARS-CoV-2 Spike protein

Spike Protein Variant	Genetic Marker	Effect	UK B 1.1.7	ZA 501.V2	DK mink V
N501Y	A23063T	RBD (stronger ACE binding)	X	X	
K417N	G22813T	RBD (ACE binding domain)		X	
Y453F	A22920T	RBD (weaker ACE binding)			X
D614G	A23403G	RBD (stronger ACE binding)	X	X	X
E484K	G23012A	RBD (ACE binding domain)		X	
P681H	C23604A	furin cleavage site	X		
del HV69/70	**del21765-770**	**evasion of immune response**	**X**		**X**

2.1) qPCR-based Detection

The first step in the discrimination between wildtype and mutant SARS-CoV-2 is the conversion of viral RNA into cDNA. Afterwards, the viral target sequences simultaneously amplified in one reaction with amplification monitored in real time using fluorescently labelled probes:

upon incorporation into the newly amplified DNA strands, the fluorophore is released and an increase in fluorescence signal can be observed.

With **PHOENIXDX® SARS- COV-2 MUTANT SCREEN [DEL HV69/70]**, discrimination between the viral targets is achieved using of two different fluorophores that are detected in two different channels: FAM™ for the wildtype virus and HEX/VIC for the mutant virus.

Due to the intrinsic mutation rate of viruses, 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 for any of the viruses should always be confirmed through complementary methods and additional analysis in an independent laboratory.

PHOENIXDX® SARS- COV-2 MUTANT SCREEN [DEL HV69/70] is compatible with every qPCR cycler with calibrated FAM™ and HEX/VIC channel.

The kit contains 2 target positive controls (**TPC**, one with the wildtype target sequence and one with the mutant sequence) to verify that the PCR assays are functional.

2.2) MATERIALS PROVIDED

QUANTITY AND VOLUME	COMPONENT
1x 100 µl	20X RT Enzyme Mix
1x 400 µl	5X MTS Buffer
1x 100 µl	WT/del HV69/70 Assay Mix
1x 100 µl	TPC SC2-WT
1x 100 µl	TPC SC2-Mutant

2.3) ADDITIONAL MATERIALS REQUIRED

- Suitable means & equipment for nucleic acid extraction (see chapter 3.4)
- Real-time PCR detection system equipped for FAM™ and HEX/VIC detection
- Adjustable pipettes & fitting filtered pipette tips
- Nuclease-free water
- Appropriate PSA & workspaces for working with potentially infectious samples
- Surface decontaminants such as DNAZap™ (Life Technologies), DNA Away™ (Fisher Scientific), RNAse Away™ (Fisher Scientific), 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 **WT/del HV69/70 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 (see tubes).

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 Mutant Screen [del HV69/70]** 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, bronchoalveolar 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.

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 the **PhoenixDx® SARS- CoV-2 Mutant Screen [del HV69/70]** assays.

Do not use specimens if

- they were not kept at 2-4°C (≤ 4 days) or frozen at -70°C or below.
- 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: 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 Mutant Screen [delHV69/70]** 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 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 **10 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 MTS Buffer	4 µl
WT/del HV69/70 Assay Mix	1 µl
Nuclease-free dH_2O	4 µl
isolated sample RNA / TPC SC2-WT/SC2-Mutant / NTC	10 µl / 10 µl / 10µl 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. An example setup is given in **Fig 2).**
   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
Pre-PCR Read[1]	1	50°C	1 minute
Reverse Transcription	1	50°C	5 minutes
Initial Denaturation	1	95°C	5 minutes
Amplification	45	95°C	5 seconds
		57°C[2]	45 seconds
Post-PCR Read[1]	1	50°C	1 minute

[1] Using the genotyping/ allelic discrimination option of your device with pre- and post-PCR reads at 50°C are highly recommended.
[2] Enable Data Collection for **FAM™** (wildtype), **HEX/VIC** (del HV69/70 mutant) and **ROX** for passive reference if required.

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

- **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 wildtype SARS-CoV-2, the FAM™ channel must give a positive Ct value.**

- **For a sample to be considered positive for del HV69/70 SARS-CoV-2, the HEX/VIC channel must give a positive Ct value.**

- **If no amplification signal in neither the FAM™ nor HEX/VIC channel is observed for any assay, PCR was inhibited.** Check reaction setup and device settings and repeat the RNA extraction if necessary. Results are invalid and cannot be interpreted.

- **When using the TPC, a positive Ct in the FAM™ (TPC SC2-WT) or HEX/VIC channel (TPC SC2-Mutant) must be observed. The Ct values for the TPC should be < 35 cycles.** If the Ct value does not correspond to the expected value or not all assays are tested positive, PCR was compromised. Check the reaction setup and PCR device settings and repeat the reactions. Repeated freeze and thaw cycles of the TPC can compromise its quality resulting in late Ct values.

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. If amplification in sample reactions seems to have failed, check if the TPC reactions are displayed simultaneously.

Table 2 Interpretation of amplification results with PhoenixDx® SARS- CoV-2 Mutant Screen [del HV69/70]

FAM™	HEX/VIC	Result
/	/	The sample does not contain **SARS-CoV-2 RNA, wildtype or del HV69/70 mutant**. The sample is considered **negative for both viruses.**
+	/	Spike Variant without deletion at 69/70 was detected. The sample is **positive for wildtype SARS-CoV-2**.
/	+	Spike Variant delHV69/70 was detected. The sample is **positive for mutant delHV69/70 SARS-CoV-2 (for example UK or DK variant)**.
+	/	Expected result for the **TPC SC2-WT.**
/	+	Expected result for the **TPC SC2-Mutant**.

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 sequences may result in failure to detect the target sequence.
- Results must always be interpreted in consideration of all other data gathered from a sample. Interpretation must be performed by personnel trained and experienced with this kind of experiment.
- 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 MUTANT SCREEN [DEL HV69/70]** is tested against predetermined specifications to ensure consistent product quality.

7) NON-CLINICAL PERFORMANCE EVALUATION

The analytical LOD (Limit of Detection) for the target sequences was determined by serial dilution of wildtype SARS-CoV-2 RNA and del HV69/70 SARS-CoV-2 RNA.

LOD (WT) >50 copies / 20 µl PCR reaction

LOD (del HV69/70) >50 copies / 20 µl PCR reaction

8) TRADEMARKS

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

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9) Literature

Preliminary genomic characterisation of an emergent SARS-CoV-2 lineage in the UK defined by a novel set of spike mutations. Rambaut et al., 2020

www.ecdc.europa.eu/en/publications-data/threat-assessment-brief-rapid-increase-sars-cov-2-variant-united-kingdom

The circulating SARS-CoV-2 spike variant N439K maintains fitness while evading antibody-mediated immunity. Thomson et al., 2020

Mutations in SARS-CoV-2 spike protein and RNA polymerase complex are associated with COVID-19 mortality risk. Hahn et al., 2020

10) Technical Assistance

For ordering, questions or technical support, contact Procomcure Biotech:

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11) Symbol Definition (Manual & Packaging)

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