PhoenixDx® ***2019-nCoV***

for research use only

qualitative RT-PCR-based detection of 2019-nCoV

INSTRUCTIONS FOR USE

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1) Intended Use

This document describes the use of real-time RT-PCR assays for the *in vitro* qualitative detection of 2019-Novel Coronavirus (2019-nCoV) in respiratory specimens and sera. The 2019-nCoV primer and probe sets are designed for the specific detection of 2019-nCoV.

The kits follow CDC's and WHO's latest detection guidelines.

2) PhoenixDx® Detection System

PhoenixDx® 2019-nCoV is a real-time RT-PCR-based detection system for the 2019 Wuhan coronavirus (**2019-nCoV**). 2019-nCoV 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® 2019-nCoV detects the presence of 3 different and highly specific gene sequences of 2019-nCoV: E gene, N gene and RdRP gene. All 3 assays must be tested positive to confirm the sample as 2019-nCoV-positive.

Additionally, a non-infectious positive control and a negative human extraction control are included. The positive control is used to confirm functionality of the assays and overall PCR performance, the negative human extraction control is included to evaluate the quality of the RNA isolation independently from the 2019-nCoV assays.

2.1) qPCR-based Detection of 2019-nCoV

The first step in the detection of 2019-nCoV is the conversion of viral RNA into cDNA. Afterwards, the target sequences unique for 2019-nCoV are specifically amplified with amplification monitored in real time through the use of fluorescently labelled probes: upon incorporation into the newly amplified DNA strands, the fluorophore (FAM™) is released and an increase in fluorescence signal can be observed.

Due to the intrinsic mutation rate of coronaviridae, 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. **PhoenixDx® 2019-nCoV** addresses this issue by using 3 detection assays on 3 different target sequences to minimize the chance false-negative results caused by an altered target sequence.

If samples are tested negative in one or more assays, additional complementary testing may be required. The original target sequences for 2019-nCoV are included as a non-infectious target positive control (**TPC**) to check the integrity of the detection assays.

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

PRODUCT	SIZE	SKU
PHOENIXDX® 2019-NCOV	100 rxn / 20 µl	PCCSKU15259

2.2) MATERIALS PROVIDED

COMPONENT
50X VitaScript™ Reverse Transcriptase
PhoenixDx® 2X qPCR Mastermix E
PhoenixDx® 2X qPCR Mastermix N
PhoenixDx® 2X qPCR Mastermix RdRP
PhoenixDx® 2X qPCR Mastermix (HEC)
2019-nCoV Target Positive Control **(TPC)**
Negative Human Extraction Control **(HEC)**
Nuclease-free dH_2O

2.3) ADDITIONAL MATERIALS REQUIRED

- Suitable means & equipment for nucleic acid extraction (see chapter 3.4)
- Real-time PCR detection system equipped for FAM™ detection
- Adjustable pipettes & fitting filtered pipette tips
- 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.
- Protect the assay mixes 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 specimen.
- 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 (2019-nCoV) 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® 2019-nCoV** is restricted to trained laboratory personnel only.

3.2) Specimen

Only use appropriate specimen 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 acceptable

3.3) Serum Specimen Handling and Storage

- Specimen can be stored at 4°C for up to 72 hours after collection.
- If a delay in extraction is expected, store specimen at -70°C or lower.
- Extracted nucleic acids should be stored at -70°C or lower.

Do not use specimen 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 specimen.

- Suitable nucleic acid extraction systems that have been successfully used in combination with **PHOENIXDX DETECTION KITS** include: 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, and Roche MagNA Pure 96 DNA and Viral NA Small Volume Kit, and Invitrogen ChargeSwitch® Total RNA Cell Kit.
- Store and keep residual specimen and extracted nucleic acids at -70°C.
- Only thaw the number of specimen extracts 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.
- It may be possible to use patient samples directly, depending on the sample type. However, this may require a prior lysis step and titration of the amount on sample that can be used without inhibiting the reaction. This procedure has not been validated, use of isolated RNA is recommended.

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® 2019-NCOV** 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) For first tests, prepare a dilution series of your RNA to determine your ideal concentration window.
6) 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. Always include the assay for the negative human extraction control **(HEC)** to evaluate the quality of your RNA isolate.
   c. When using the provided target positive control **(TPC)**, use **1 µl / reaction** and add nuclease-free dH_2O to 20 µl.
   d. > 2 replicates / sample are strongly recommended.
   e. Prepare enough mastermix for all planned reactions. It is recommended to prepare mastermix for 2 additional reactions to compensate for pipetting inaccuracies.
   f. Distribute the mastermix to your strips/plate. An example setup is given in **Fig 1).**

COMPONENT	VOLUME
50X VitaScript™ Reverse Transcriptase	0.4 µl
PhoenixDx® 2X qPCR Mastermix (E / N / RdRP / HEC)	10 µl
isolated sample RNA / TPC / HEC	X µl
Nuclease-free dH_2O	to 20 µl

7) 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.

Fig. 1 Example pipetting scheme for the distribution of mastermixes with the individual assay mixes.

	1	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**
A	E	E	E	E	E	E	E	E	E	E	E	E
B	N	N	N	N	N	N	N	N	N	N	N	N
C	RdRP	RdRP	RdRP	RdRP	RdRP	RdRP	RdRP	RdRP	RdRP	RdRP	RdRP	RdRP
D	HEC	HEC	HEC	HEC	HEC	HEC	HEC	HEC	HEC	HEC	HEC	HEC
E												
F												
G												
H												

Fig. 2 Example pipetting scheme for the addition of samples. The bottom half of the plate could be used for replicates with an identical setup.

	1	2	3	4	5	6	7	8	9	10	11	12
A	NTC	S1	S2	S3	S4	S5	S6	S7	S8	S9	S10	TPC
B	NTC	S1	S2	S3	S4	S5	S6	S7	S8	S9	S10	TPC
C	NTC	S1	S2	S3	S4	S5	S6	S7	S8	S9	S10	TPC
D	NTC	S1	S2	S3	S4	S5	S6	S7	S8	S9	S10	TPC
E												
F												
G												
H												

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

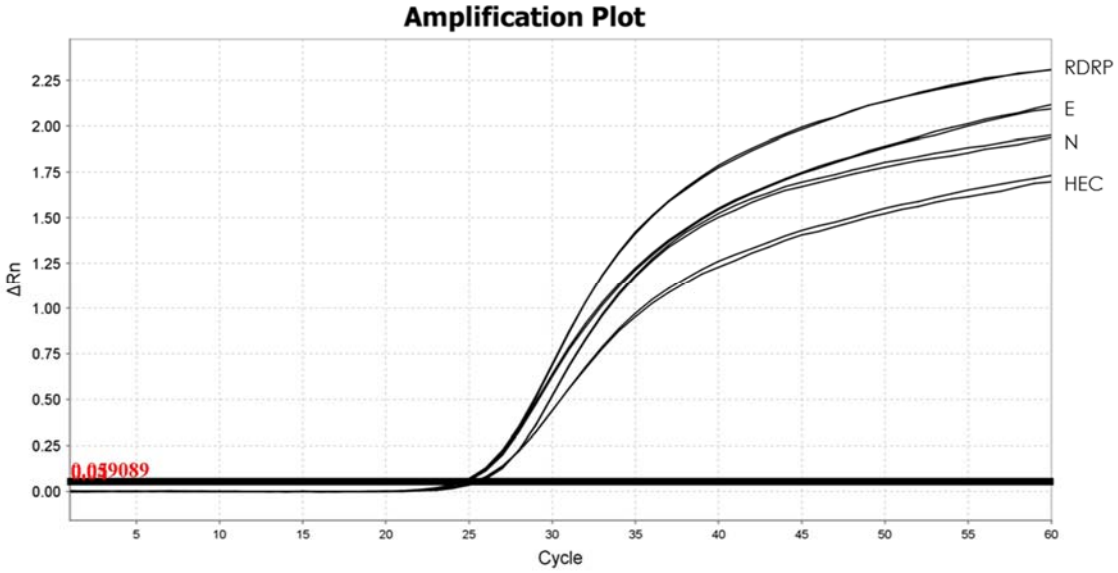
Step	**Cycles**	**Temperature**	**Duration**
Reverse Transcription	1	45°C	20 minutes
Initial	1	95°C	10 minutes
Amplification	45	95°C	15 seconds
		58°C[1]	45 seconds

[1] Enable Data Collection for **FAM™.** If required, set Passive Reference to **ROX™**.

9) 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 & TROUBLESHOOTING

Example Result



- **dH₂O controls (NTC) must not give a positive Ct for any assay**. If they do, the reaction was contaminated with sample RNA / DNA. Decontaminate equipment and workspace and repeat the reactions.

- **For a sample to be considered positive for 2019-nCoV, all 3 assays (E / N / RdRP) must give positive Ct values.** If the **HEC** fails to amplify, the sample must still be considered positive.

- **For a sample to be considered negative for 2019-nCoV, none of the 3 assays (E / N / RdRP) must give positive Ct values.** The **HEC** must give a positive Ct value (< 35 cycles) for these samples to ensure that sample material of suitable quality was present.

- **All reactions containing RNA isolate must give positive Ct values for the HEC assay. The Ct values should be < 35 cycles.** Failure to amplify the negative human extraction control indicates a flawed RNA extraction or loss of RNA isolate due to RNAse contamination. The sample is not sufficient, results cannot be interpreted.

- **When using the TPC for 2019-nCoV, a positive Ct for all 3 assays must be observed. The Ct value 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.

- **If no amplification signal 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.

Fig. 3 Interpretation of amplification results with PhoenixDx 2019-nCoV

E	N	RdRP	HEC	Interpretation
+	+	+	+	All 3 target sequences for 2019-nCoV and the **HEC** were amplified. The sample is considered positive for 2019-nCoV.
/	/	/	+	Only the target sequence for the HEC was amplified. The sample is considered negative for 2019-nCoV.
+	+	/	+	Indicates other Sarbecovirus (SARS or SARS-related Coronavirus) but not Wuhan / 2019-nCoV.
/	/	/	/	PCR was inhibited, results are invalid.

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.
- 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.

6) Trademarks

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

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7) Technical Assistance

For questions or technical support, contact Procomcure Biotech:

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