PhoenixDx® **SARS-CoV-2 N501Y Multiplex**

First line screening of SARS-CoV-2 and detection of N501Y variants

for diagnostic use

qualitative RT-PCR-based detection of SARS-CoV-2 and N501Y SARS-CoV-2

INSTRUCTIONS FOR USE

96 Tests

REF **PCCSKU15275**

v 1.0

CE IVD

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1) Intended Use

PhoenixDx® SARS-CoV-2 N501Y Multiplex is a real-time RT-PCR-based diagnostic test for the *in vitro* qualitative detection and discrimination of SARS-CoV-2 and the N501Y mutant in respiratory specimens and sera from patients who meet COVID-19 clinical and/or epidemiological criteria.

PhoenixDx® SARS-CoV-2 N501Y Multiplex 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 N501Y Multiplex** is intended for use by clinical laboratory personnel specifically instructed and trained in the techniques of real-time PCR and *in vitro* diagnostic procedures.

The kits follow CDC's and WHO's latest detection guidelines (03/2020).

2) PhoenixDx® Detection System

PhoenixDx® SARS-CoV-2 N501Y Multiplex 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 N501Y Multiplex detects a target sequence highly specific for SARS-CoV-2 (N gene), mutant N501Y SARS-CoV-2 (S gene) and one sequence specific for human RNA (RNAse P) serving as a human extraction control (**HEC**). Additionally, a non-infectious target positive control (**TPC**) is included. The positive control is used to confirm functionality of the assays and overall PCR performance, 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 and discrimination of SARS-CoV-2 and mutant N501Y 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 N501Y MULTIPLEX is compatible with every qPCR cycler with calibrated Cy5, ROX and FAM™.

2.2) MATERIALS PROVIDED

QUANTITY AND VOLUME	COMPONENT
1x 100 µl	20X RT Enzyme Mix
1x 400 µl	5X MTM Buffer
1x 100 µl	N501Y 3Plex Mix
1x 100 µl	N501Y 3Plex 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 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 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 aliquotes if required).
- Protect the **N501Y 3Plex 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 N501Y MULTIPLEX** 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.

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 N501Y MULTIPLEX.**

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 N501Y Multiplex** 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 MTM Buffer	4 µl
N501Y 3Plex Mix	1 µl
Nuclease-free dH_2O	4 µl
Isolated sample RNA / N501Y 3Plex TPC / 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.
   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	45	95°C	5 seconds
		57°C[1]	45 seconds

[1] Enable Data Collection for **FAM™** (N501Y), **ROX** (SARS-CoV-2) and **Cy5** (**HEC**). 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™ channel 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 ≥ 30.

- **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 22-29. 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 N501Y SARS-CoV-2, the FAM™ channel must give a positive Ct value.** Amplification of the HEC in the **Cy5 channel** is expected around Ct 22-29. 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.

- **If amplification in the FAM™ channel and 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™ channel nor the ROX or Cy5 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.

- **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 22-29.** 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.

- **When using the TPC, a positive Ct in the FAM™ channel and in the ROX channel 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 1 Interpretation of amplification results with PhoenixDx® SARS-CoV-2 N501Y Multiplex

FAM™	ROX	Cy5	Interpretation
+	+	+	**N501Y SARS-CoV-2 and SARS-CoV-2** target sequences & **HEC** were amplified. The sample is considered positive for N501Y SARS-CoV-2.
/	+	+	**SARS-CoV-2** target sequence & **HEC** was amplified. The sample is considered positive for SARS-CoV-2.
+	/	+	**N501Y SARS-CoV-2** target sequence was 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™	ROX	Cy5	Interpretation
/	/	+	Only the target sequence for the **HEC** was amplified. The sample is considered negative for SARS-CoV-2.
≥ 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 N501Y 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.
/	/	/	PCR was inhibited, results are invalid.
+	+	+	Expected Result for the **TPC**.

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™ channel 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 ≥ 30.
- 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

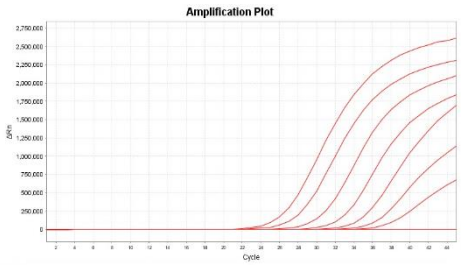
In accordance with Procomcure Biotech GmbH's EN ISO 13485-certified Quality Management System, each lot of **PhoenixDx® SARS-CoV-2 N501Y Multiplex** is tested against predetermined specifications to ensure consistent product quality.

7) Analytical Sensitivity & Linearity

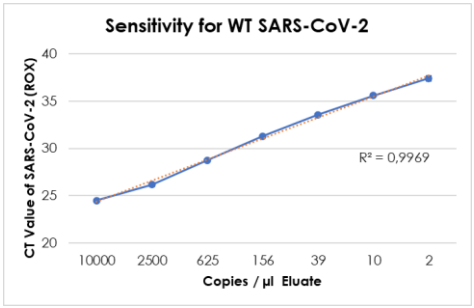
The LOD95(Limit of Detection) defines the number of target sequences (copy number) that can be detected in ≥ 95% of reactions. The LOD95 was determined by testing a serial dilution of isolated SARS-CoV-2 RNA with 7 concentrations in 24 replicates per concentration for the WT and with 4 concentrations in 24 replicates per concentration for the N501Y variant.

SARS-CoV-2

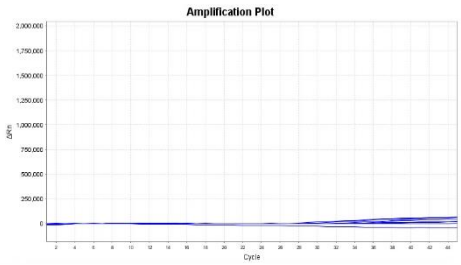
ROX Channel



Sensitivity & Linearity



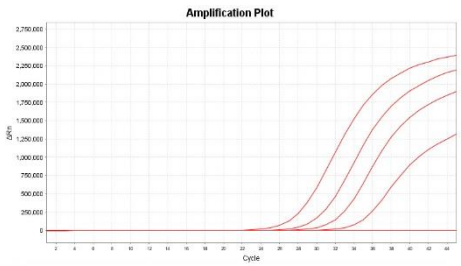
FAM™ Channel



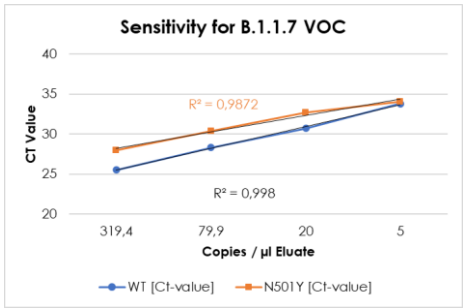
The LOD95 for WT SARS-CoV-2 was determined to be 2 copies / µl RNA eluate.

N501Y SARS-CoV-2 (B.1.1.7 lineage)

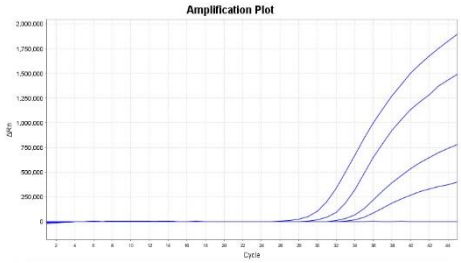
ROX Channel



Sensitivity & Linearity



FAM™ Channel



The LOD95 for SARS-CoV-2 was determined to be 5 copies / µl RNA eluate.

8) Clinical Data

The performance of **PhoenixDx® SARS-CoV-2 N501Y Multiplex** was tested in a paired comparison using collected nasopharyngeal swabs. **PhoenixDx® SARS-CoV-2 N501Y Multiplex** was evaluated using clinical samples (60 samples for WT SARS-CoV-2, 46 samples for N501Y SARS-CoV-2) collected from patients with signs and symptoms of an upper respiratory infection against a validated CE IVD reference kit with the intended use of detecting SARS-CoV-2 RNA. RNA isolation was automatically performed using 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 N501Y Multiplex	
		positive	negative
Positive	50	A= 50	B= 0
Negative	10	C= 0	D= 10
Clinical sensitivity = [a/(a+c)] ×100 = [50/(50+0)] x100 =			100%
Clinical specificity = [d/(b+d)] ×100 = [15/(0+15] x100 =			100%

*For Ct values ranging from 18-37

Reference Method	n	PhoenixDx® SARS-CoV-2 N501Y Multiplex	
		positive	negative
501N SNP	22	A= 22	B= 0
N501Y SNP	24	C= 0	D= 24
Clinical sensitivity = [a/(a+c)] ×100 = [22/(22+0)] x100 =			100%
Clinical specificity = [d/(b+d)] ×100 = [24/(0+24] x100 =			100%

*For Ct values ranging from 18-30

9) Trademarks

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

Registered names, trademarks, etc. used in this document, even if not specifically marked as such, are not to be considered unprotected by law.

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) Compatible Cycler

Applied Biosystems 7500, Applied Biosystems 7500FAST, Applied Biosystems QuantStudio 5, Applied Biosystems Viia7, Biorad CFX.

[for all listed cyclers with 96-well block and valid calibration for FAM, ROX and Cy5]

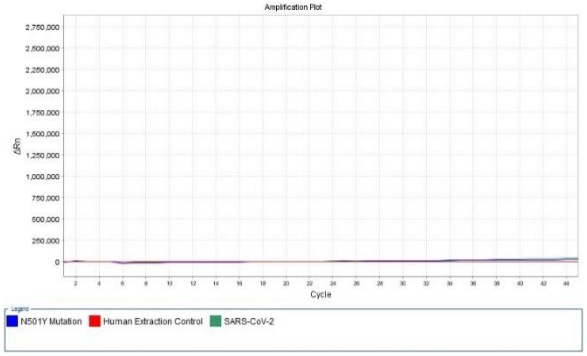
12) Example Results

PCR setup was performed as described in this IFU. PCR run was performed on the Applied Biosystems 7500FAST qPCR Cycler.

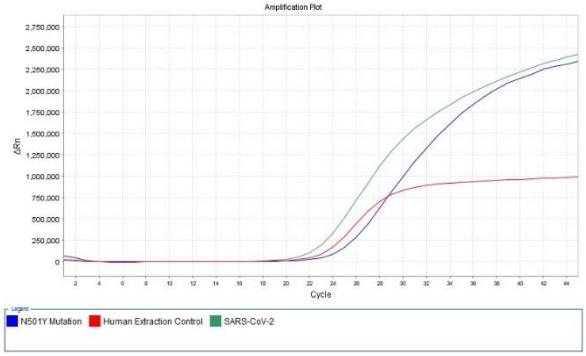
Blue: N501Y Mutation (FAM channel)

Red: Human Extraction Control (Cy5 channel)

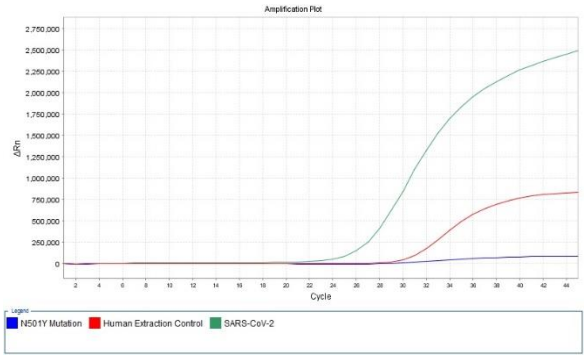
Green: SARS-CoV-2 (ROX channel)



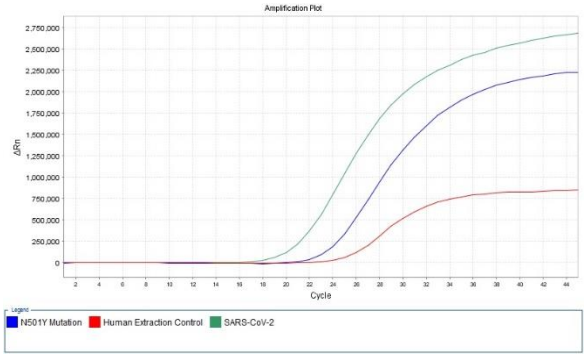
Negative Control (water)



Positive Control (N501Y 3Plex TPC)



Positive sample with wildtype SARS-CoV-2



Positive sample with UK variant SARS-CoV-2

13) Technical Assistance

For questions or technical support, contact Procomcure Biotech:

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14) 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

NOTES

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