DIAGNOVITAL

SARS-CoV-2 P681H Mutation Detection Kit

Qualitative RT-PCR-based detection of SARS-CoV-2 P681H Mutation

For Research use only. Not for use in diagnostic procedures.

RUO

REF Σ

090R17025 25 test

090R17025 50 test

090R17100 100 test

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RTA Laboratuvarları
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Intended Use

DIAGNOVITAL® SARS-CoV-2 P681H Mutation Detection Kit is a real-time RT-PCR-based test for the detection and differentiation of SARS-CoV-2 P681H mutation in respiratory specimens. This kit is designed to run only samples that have previously proven to be SARS-CoV-2 positive.

DIAGNOVITAL® SARS-CoV-2 P681H Mutation Detection Kit detects P681H mutation in extracted SARS-CoV-2 RNA from nasopharyngeal and oropharyngeal swab samples during infection. Positive results indicate the presence of SARS-CoV-2 RNA and allow P681H in the Spike protein. Spike protein of wildtype SARS-CoV-2 has Proline (P) amino acid at 681th location whereas variant of SARS-CoV-2 has Histidine (H) amino acid at 681th location.

The P681H mutation was discovered in samples from around the world as early as March 2020, including Nigeria, Hawaii, and, most recently, three independent variants in New York, and it also characterizes the globally spreading B.1.1.7 VOC. The P681H mutation is thought to increase virus transmissibility by facilitating a conformational change in the S protein after protease activity at the cell membrane because it is part of a furin and furinlike protease cleavage site at the junction of the spike protein receptor-binding (S1) and fusion (S2) domains. Furthermore, the A.23.1 variant, which has been identified as the dominant lineage in Uganda, and the B.1.617 family of variants, which has been identified in India, both acquired the P681R mutation in the same position.

Product Description

DIAGNOVITAL® SARS-CoV-2 P681H Mutation Detection Kit is a real-time RT-PCR-based detection and differentiation system for SARS-CoV-2 P681H mutation. **This kit is designed to run only on samples that have previously proven to be SARS-CoV-2 positive.**

DIAGNOVITAL® SARS-CoV-2 P681H Mutation Detection Kit also detects the presence of a sequence specific human target (RNAseP) serving as a human extraction control (HEC) in addition to the wild type (P681) and the mutant (H681) sequences in different channels.

REAL TIME PCR-BASED DETECTION OF SARS-CoV-2

The first step in the detection of SARS-CoV-2 P681H mutation is the conversion of viral RNA into cDNA. Afterwards, the viral target sequences and the RNAseP (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.

With **DIAGNOVITAL® SARS-CoV-2 P681H Mutation Detection Kit** differentiation between the viral targets is achieved through the use of two different fluorophores that are detected in three different channels: FAM™ for wild type SARS-CoV-2 P681, HEX/VIC for H681 mutation and the RNAseP (HEC) is detected in the Cy5 channel.

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.

DIAGNOVITAL® SARS-CoV-2 P681H Mutation Detection Kit is validated with Applied Biosystems Quant Studio 5 Dx and compatible with BioRad CFX96, Applied Biosystems Quant Studio 5 Real-Time PCR Systems calibrated FAM™, HEX/VIC and Cy5 channels.

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Materials Provided

Table 1: DIAGNOVITAL® SARS-CoV-2 P681H Mutation PCR Kit contents

	Reagents	Quantity and Volume (25 tests)	Quantity and Volume (50 tests)	Quantity and Volume (100 tests)
1	PCR Master Mix	1 × 375 µl	1 × 750 µl	1 × 1500 µl
2	Positive Control	1 × 38 µl	1 × 75 µl	1 × 150 µl
3	Nuclease-free dH_2O	1 × 38 µl	1 × 75 µl	1 × 150 µl

IMPORTANT! The table above reflects the standard kit color scheme. Due to supplier issues during the COVID-19 crisis, individual tube cap colors may be substituted due to availability. Always check the labeling of the reagent prior to use.

Additional Materials Required

- Suitable means & equipment for nucleic acid extraction
- Real-time PCR detection system equipped for FAM™, HEX/VIC and Cy5 detection
- Adjustable pipettes & fitting filtered pipette tips
- Appropriate personal protective equipment & 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, master mixes etc.
- Real-Time PCR reaction tubes/plates/capillaries
  For **BIO-RAD CFX96**: Hard-Shell Thin-Wall 96-Well Skirted PCR Plates with Bar Codes (BIO-RAD, Cat#: HSP-9955), Hard-Shell Thin-Wall 96-Well Skirted PCR Plates (BIO-RAD, Cat#: HSP-9655), Microseal 'B' Adhesive Seals, optically clear (BIO-RAD, Cat#: MSB-1001), Individual PCR Tubes, Low Tube Strips, 8-tubes strip, 0.2 ml Low Profile, White (BIO-RAD, Cat#: TLS0851), Flat Cap Strips, Optically Clear, 8-cap strip, 0.2 ml (BIO-RAD, Cat#: TCS0803).

  For **Applied Biosystems Quant Studio 5 Dx- Quant Studio 5 Real-Time PCR System,** MicroAmp® Optical 96-Well Reaction Plate (Thermo Fisher, Cat#: 4306737), MicroAmp® Optical Adhesive Film (Thermo Fisher, Cat#: 4311971), MicroAmp® Optical 8-Tube Strip, 0.2 mL (Thermo Fisher, Cat#: 4316567), MicroAmp® Optical 8-Cap Strips (Thermo Fisher, Cat#: 4323032)

- Suitable storage options for reagents and specimen (4°C, -20°C, -70°C)

Storage

- Store all components at -15°C /-25°C and avoid more than 3 freeze and thaw cycles.
- Protect the qPCR master mix from light as prolonged exposure can diminish the performance of the fluorophores.
- If the kit components have been damaged during transport, contact RTA Laboratories. 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.

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Performance Characteristics

Analytical sensitivity

The analytical sensitivity or detection limit for nucleic acid–based assays shall be expressed by the 95% positive cutoff value. This is the analyte concentration where 95% of test runs give positive results following serial dilutions using an reference material. In this study analytical sensitivity was analyzed by use of a dilution series of VIRCELL AMPLIRUN® SARS-CoV-2 RNA CONTROL (21MBC137002-R) for wild type channel and VIRCELL AMPLIRUN® SARS-CoV-2 B.1.1.7 (21MBC138001-R) RNA CONTROL for mutant channel. Dilutions were made by negative clinical RNA sample. Each dilution was tested on with 23 replicates. QuantStudio 5-DX Real-time PCR Systems was used for amplification, signal detection and analysis of, the results. Probit analysis was done by IBM SPSS Statistics 27 program. The results are shown in the table below.

Table 2: DIAGNOVITAL P681H Mutation Detection - Limit of Detection (LoD) values and 95 % confidence ranges

Target gene	Limit of Detection (copies/ml)	95% confidence lower limit	95% confidence upper limit
P681	5460.957	4443.903	7453.119
H681	6085.187	5343.253	11423.383

Diagnostic specificity

A total of 24 clinical samples that were collected from patients with Covid symptoms were analyzed by **DIAGNOVITAL® SARS-CoV-2 P681H Mutation Detection Kit** and the results were compared against next generation sequencing analysis. 24 of them were found to be positive for the P681H mutation. The negative percent agreement (NPA) of DIAGNOVITAL® SARS-CoV-2 P681H Mutation Detection Kit with respect to NGS is 100%. All of the Internal Controls (RNAseP) have been tested positive.

Table 3. DIAGNOVITAL® SARS-CoV-2 P681R Mutation Detection Kit –NGS qPCR correlation study

		Comparator (NGS and qPCR)	
NP swab		Mutant	Wild Type
DIAGNOVITAL SARS-CoV-2 P681H Mutation Detection Kit	R681	0	0
	H681	0	24
	Total	0	24

Considerations Before Starting

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:

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- 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/lab/guidelines-clinical-specimens.html
- Biosafety in Microbiological and Biomedical Laboratories 6th edition available at http://www.cdc.gov/biosafety/publications/.

- The use of DIAGNOVITAL® SARS-CoV-2 P681H Mutation Detection Kit 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.

Specimens

Only use appropriate specimens for testing, such as:

- Respiratory specimens including nasopharyngeal / oropharyngeal.
- Swab specimens should be collected only on swabs with a synthetic tip (such as polyester or Dacron®) with plastic shafts. Swabs with calcium alginate or cotton tips with wooden shafts are not acceptable.

Specimens - Handling and Storage

- Specimens can be stored at 2-8°C for up to 72 hours after collection.
- If a delay in extraction process is expected, store specimens at -20°C or lower.
- Extracted nucleic acids should be stored at -20°C or lower.

Do not use specimens if

- they were not kept at 2-8°C (≤ 4 days) or frozen at -20°C or lower.
- 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.

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Sample Preparation

- 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.
- **DIAGNOVITAL® SARS-CoV-2 P681H Mutation Detection Kit** is validated with RTA Viral RNA Isolation Kit.
- **DIAGNOVITAL® SARS-CoV-2 P681H Mutation Detection Kit** is compatible with Tianlong Generotex96 Extraction System and QIAamp® MinElute Virus Spin Kit.
- *Validated Real Time PCR systems*: Applied Biosystems Quant Studio 5 Dx.
- *Compatible Real Time PCR systems* BioRad CFX96, Applied Biosystems Quant Studio 5 Real-Time PCR Systems.
- Store and keep residual specimens and extracted nucleic acids at -20°C or -80°C.
- Only thaw the number of specimen extracts that will be tested in a single day.
- Do not freeze/thaw extract 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.

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 **DIAGNOVITAL® SARS-CoV-2 P681H Mutation Detection Kit** 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 (via microcentrifuge).
5. The **PCR Master Mix** provided with **DIAGNOVITAL® SARS-CoV-2 P681H Mutation Detection Kit** is ready to use. One reaction will be prepared for each sample. A separate reaction should be prepared for Negative Control (NTC) and Positive Control (TPC).

Component	**Volume (µl)**
PCR Master Mix	15
RNA Isolate/ TPC/ NTC	5
Total	**20**

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6. Distribute **15 µl** PCR Master Mix to your strips/plate and add **5 µl your samples**. (An example setup is given in **Fig1**).

	1	2	3	4	5	6	7	8	9	10	11	12
A	S1	S9	S17	S25	S33	S41	S49	S57	S65	S73	S81	TPC
B	S2	S10	S18	S26	S34	S42	S50	S58	S66	S74	S82	S89
C	S3	S11	S19	S27	S35	S43	S51	S59	S67	S75	S83	S90
D	S4	S12	S20	S28	S36	S44	S52	S60	S68	S76	S84	S91
E	S5	S13	S21	S29	S37	S45	S53	S61	S69	S77	S85	S92
F	S6	S14	S22	S30	S38	S46	S54	S62	S70	S78	S86	S93
G	S7	S15	S23	S31	S39	S47	S55	S63	S71	S79	S87	S94
H	S8	S16	S24	S32	S40	S48	S56	S64	S72	S80	S88	NTC

Figure 1: Example pipetting scheme for the distribution of master mixes with the individual assay mixes

7. Transfer the reactions to the PCR device, then proceed according to these guidelines:

Table 4: DIAGNOVITAL SARS-CoV-2 L452R Mutation Detection Kit Thermal Profile

Step	**Cycles**	**Temperature**	**Duration**
Reverse Transcription	1	50°C	10 minutes
Initial Denaturation	1	95°C	2 minutes
Amplification	40	95°C	5 seconds
		60°C*	30 seconds

*Enable Data Collection for **FAM™** (Wild Type P681 SARS-CoV-2), **HEX/VIC** (H681 SARS-CoV-2 Mutation) and **Cy5** (HEC).

8. 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.

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Analysis & Troubleshooting

EXEMPLAR RESULTS

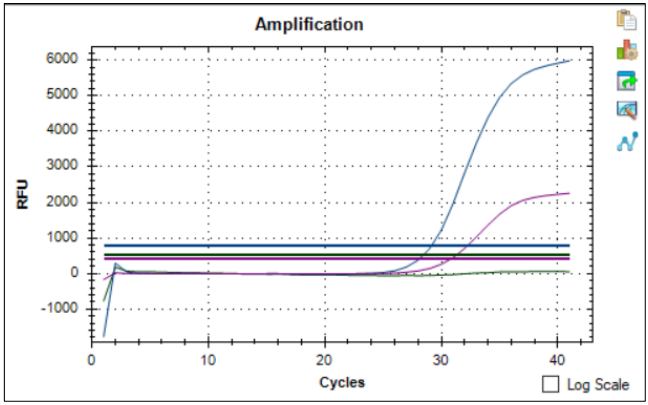

Figure 2: *Blue Curves: P681 Wild Type sample at the* **FAM** *channel, Purple Curves: internal control at the* **Cy5** *channel.*

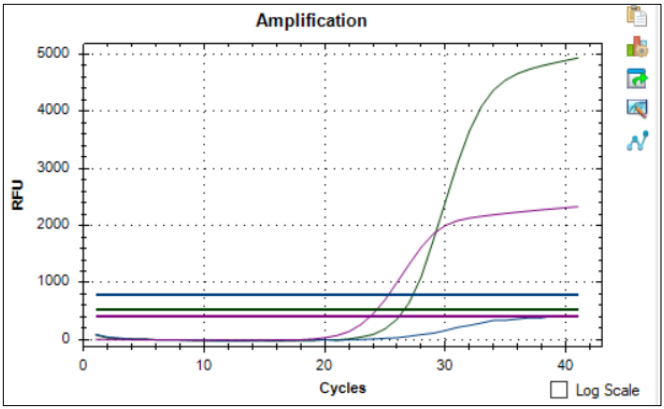

Figure 3: *Green Curves: H681 Mutant positive sample at the* **HEX** *channel, Purple Curves: internal control at the* **Cy5** *channel.*

- **dH$_2$O 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.

- **All reactions containing RNA isolate must give positive Ct values for the internal control assay. The Ct values should be ≤ 38 cycles**. Failure to amplify the internal control indicates a flawed RNA extraction or loss of RNA isolate due to RNase contamination. The sample is not sufficient, results cannot be interpreted.

- **For a sample to be considered negative for P681H mutation, the FAM™ and the Cy5 channels must give a positive Ct value ≤ 38 cycles**. Amplification of the HEC in Cy5 channel is expected around Ct 20-38.

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- **For a sample to be considered positive for P681H mutation, the HEX/VIC channel must give a positive Ct value ≤ 38 cycles**. Amplification of the HEC in Cy5 channel is expected around Ct 20-38. 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 the positive control, a positive Ct at the FAM and HEX/VIC channels must be observed. The Ct value for the positive control should be 20±3**. If the Ct value does not correspond to the expected value or the **positive control** was not tested positive, PCR was compromised. Check the reaction setup and PCR device settings and repeat the reactions. Repeated freeze and thaw cycles of the **positive control** can compromise its quality resulting in late Ct values.

- If Ct is ≤38 for each of the FAM, HEX/VIC and Cy5 channels, the result in the respective channel should be considered as **POSITIVE**, if Ct is > 38 or no value is received, the result in the relevant channel should be considered as **NEGATIVE**.

FAM	HEX/VIC	Cy5	Result
-	-	+	The sample is negative for **SARS-CoV-2**. **This kit is designed to run only on samples that have previously proven to be SARS-CoV-2 positive.** -Be sure that the sample is SARS-CoV-2 positive.
+	-	+	The sample is **negative for P681H mutation**.
-	+	+	The sample is **positive for P681H mutation.**
-	-	-	No amplification in any channel indicates flawed RNA isolation, sample degradation or PCR inhibition. Results cannot be interpreted. -RNA may be degraded during transport, extraction or storage. -Variations in the probe binding regions-sequence analysis with a different method is recommended.
+	+	-	Expected result for the ***Positive Control (TPC)***.
-	-	-	Expected result for the ***Negative Control (NTC)***.

Device Setup

For **QUANTSTUDIO5DX**, In the Home screen, create or open a template. In the New Experiment pane, click on Create New Experiment button to create a new template. In the Properties tab, enter the template information. In the Method tab, adjust the reaction volume and set up appropriate thermal profile. In the Plate tab (Quick Setup), assign plate attributes by selecting the Passive Reference from the dropdown list. In the Plate tab (Quick Setup), define and assign well attributes and select wells in the Plate Layout or the Well Table. Then assign samples and targets to selected wells.
Note: New sample or target names entered in the Quick Setup subtab are automatically populated with default values for Reporter as FAM, for Quencher as NFQ-MGB and for Task as Unknown. Edit these values in the Advanced Setup subtab. For TaqMan probes, NFQ-MGB option must be used as the quencher. Then start the run.

For **BIORAD CFX 96**, in the Software application, open the protocol from the File menu item. Create the appropriate protocol for the kit intended to be used. In the Plate tab, define and assign well attributes and select wells in the Plate Layout or the Well Table. Then assign samples and targets to selected wells. Then start the run.

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Baseline and Threshold Settings

After the run,

For **QUANTSTUDIO5DX**, click on Show Plot Settings button to change graph type from log scale to linear scale. The target can be changed in Target section. Then click on the Analysis Setting button to adjust the baseline threshold. Unclick the Automatic Threshold and Unclick the Automatic Baseline. Set the baseline start cycle to 7-8 and baseline end cycle to 20 in order to normalize the graphics.

For **BIORAD CFX 96,** threshold can be adjust according to the the ratio of FAM vs HEX signal height.

The amplification curve baseline is one of the parameters that can affect PCR results. In case the baseline is incorrectly set, a Ct value can be displayed even if no real amplification occurred. Auto threshold is used with **DIAGNOVITAL® SARS-CoV-2 P681H Mutation Detection Kit** for PCR Detection systems. If the increase of a sample in any channel is **less than 10%** of the increase of the **Positive Control** in the same channel, this increase is considered as **NEGATIVE**. In some cases, the threshold should be set manually to avoid background fluorescence. For each sample, the ratio of FAM vs HEX signal height should be checked; the signal that outweighs the other by 3-fold or more should be regarded as positive, since only one of them should amplify.

Troubleshooting

PROBLEM	POTENTIAL REASONS	SOLUTION
Negative Result for Internal Control	PCR Master Mix may not have been homogenous.	Pipetting should be performed for PCR Master Mix.
	RNA isolation may not be performed properly.	The study should be repeated from isolation.
	Isolate may contain an inhibitor.	Real Time PCR stage should be repeated by diluting the isolate 1/10.
Positive Result for NTC	Contamination may have occurred.	Contamination may have occurred from the work area to the consumable items being worked on. It is recommended to dispose of consumables and open new ones and clean the environment first with 10% NaClO solution and then with 70% Alcohol.

Limitations

- This kit is designed to run only samples that have previously proven to be SARS-CoV-2 positive.
- 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.

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- 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.

Trademarks

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Symbols


Expiry Date

Lot/ Batch

Catalog Number


Temperature Limitation


Caution


Manufacturer


Consult instructions for use or consult electronic instructions for use


Contains sufficient for (n) amount tests

Research use only.

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