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One-step RT-qPCR kit for detection of Coronavirus (COVID-19) in Humans-Single tube format

AFFIGENIX COVID-19 TEST (ACT-1™) 3 Genes MULTIPLEX KIT

Manufacturing site:

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INTRODUCTION

A novel coronavirus that is identified by Chinese researchers to be the cause of outbreak of pneumonia of unknown etiology in Wuhan city, Hubei province, China and now the pandemic disease is named as COVID-19 . This virus causes respiratory illnesses and in severe cases, organ failure and death. Early diagnosis is very crucial for disease management and control of disease spread. The CDC has recommended the application of qualitative RT-qPCR for early detection of COVID-19 virus in human clinical specimens.

PRODUCT DESCRIPTION

- AFFIGENIX COVID-19 TEST (ACT-1™) 3 Genes MULTIPLEX KIT is a qualitative robust clinical laboratory test based on real time PCR.
- The kit component includes ready to use real-time reverse transcription polymerase chain reaction mix and primer - probe sets designed to detect specific RNA sequences from individuals suspected of COVID-19 infection.
- The assay targets highly specific and conserved regions of the SARS-CoV-2 RNA such as **E gene**, **N1 gene and ORF 1ab gene** fragment for the detection of COVID-19 and RNAse P for house keeping gene as Internal control .
- Includes all reagents required to analyse RNA samples for performing RT- qPCR.
- Throughput – Can screen upto 94 samples in one single run.

PERSONAL SAFETY

- Due to highly contagious nature of the virus, all works associated with live virus sample should be performed within a BSL2 biosafety hood with BSL3 practices.
- Follow the lab biosafety guidelines provided for COVID-19 by CDC.
- All specimens and positive controls should be treated as potentially infectious and handled with caution.
- Wear proper PPEs (Personal Protective Equipment) such as gloves, head cap, face mask, eye protection and lab coats/full overall while extracting RNA from clinical specimens and also when handling kit reagents, pipettes and equipment.
- Eating, drinking, smoking, and contact with skin and eye should be avoided while handling the specimens and reagents.
- All used and unused reagents, plastic wares (reaction tubes/strips) should be packed in biohazard bags, decontaminated and disposed as per the pollution controlled board norms of the state.

RECOMMENDED WORK PRACTICES

Amplification technologies such as RT-qPCR are sensitive to the accidental introduction of PCR product from previous amplification reactions. Incorrect results (False positive) could occur if the real-time reagents used in the amplification step become contaminated by accidental introduction of amplification product (amplicon).

The following recommendations will help to prevent cross-contamination.

- Unidirectional workflow in the laboratory layout.
- Dedicated areas for sample preparation, pre-PCR assay/reaction room and PCR room.
- Use nuclease-free pipette tips with aerosol barriers for liquid handling.
- Separate, dedicated equipment (e.g., pipettes, micro centrifuges) and consumables (e.g., microcentrifuge tubes, pipette tips) for assay setup and for handling of extracted nucleic acids.
- Clean PPE and new powder-free disposable gloves are must when setting up the assay.
- All work surfaces must be disinfected thoroughly before and after completion of work.
- Microbial and nuclease contamination of samples and kit components should be prevented.
- Make sure to avoid accidental introduction of PCR product from previous amplification reactions.
- Keep reagent and reaction tubes closed as much as possible.

USAGE LIMITATIONS

- The kit should be handled by trained personnel in techniques of real-time PCR.
- Strictly follow the product insert for optimal results.
- Do not use the kit beyond the expiry date mentioned for the reagents.
- For ideal performance, store the kit under recommended conditions only.

SAMPLE TYPE

Check the CDC website for guidance on specimen collection handling and storage (https://www.cdc.gov/coronavirus/2019-nCoV/lab/guidelines-clinical-specimens.html).

- Respiratory specimens:
  Bronchoalveolar lavage, tracheal aspirate, sputum, nasopharyngeal swab and oropharyngeal swab
- Serum/plasma/blood
- Tissues

SAMPLE HANDLING AND STORAGE

- Samples collected with swabs made of calcium alginate are not acceptable.
- Hemolysed serum samples should be avoided.
- Respiratory and serum samples should be kept at 4°C for no longer than 3 days and for long term storage, store at -70 °C.
- Store the extracted RNA at -70 °C or lower.
- Follow local and national guidelines for transporting the samples.

COMPATIBLE PCR INSTRUMENTS

AFFIGENIX COVID-19 TEST (ACT-1™) 3 Genes MULTIPLEX KIT is compatible with Applied Biosystems instrument models 7500, Quant studio 3 and 5.

RECOMMENDED RNA EXTRACTION KIT

QIA AMP RNA Mini prep kit or equivalent.

ASSAY setup requirements

Primary sample	A respiratory sample (sample from nose or throat, swab) in viral transport media (VTM)
Storage	All respiratory specimens should be kept at 4 deg C for no longer than 4 days.
Requisitions	Test requisitions should be accompanied with samples.
Rejection criteria	Low sample volume, contaminated sample, sample not collected in proper container
Specimen preparation reagents (RNA isolation)	QIA Amp Viral RNA mini kit, Magmax or equivalent.
Amplification and detection reagents	**AFFIGENIX COVID-19 TEST (ACT-1™) 3 Genes MULTIPLEX KIT**

Material required but not Supplied

- Vortex mixer
- Micro centrifuge
- Micropipettes (2, 10, 100, 200 and 1000 µL).

- Racks for micro centrifuge tubes
- 2X 96 well 20 deg C cold block
- 7500 real-time PCR systems/Applied Biosystems Step one RT-qPCR / Applied Biosystems QS3 &5 or equivalent.
- Nuclease-free water
- Surface sanitizer
- QIA AMP Viral RNA mini kit or equivalent
- Aerosol barrier pipette tips
- 1.5mL centrifuge tube DNase and RNase free PCR vials
- Refrigerator +2 to +8 deg C.
- Freezer : -Below -10 deg C
- Laminar air flow hood
- 96- 100 % Ethanol
- PPEs

KIT COMPONENTS FOR REAL TIME PCR AMPLIFICATION

Component Name	Component Description	Storage
Multiplex PCR Mix	One step Multiplex PCR master mix	<-10 deg C
Detection Mix	Primer and probe mix for E gene, N1 and ORF 1ab and RNase P	<-10 deg C
Positive template control (PTC)	Synthetic RNA template for E, N1, ORF 1ab and RNase P	<-10 deg C

REAGENT STORAGE, HANDLING AND STABILITY

- Store all the kit components at temperature lower than -10°C.
- AFFIGENIX COVID-19 TEST (ACT-1™) 3 Genes MULTIPLEX KIT reagents are stable with shelf-life at 2-8°C for 5 days and at less than -10°C for 12 months.
- Immediately store the kit components to their respective storage temperature after use.
- Try to minimize the freeze thaw cycles (2X) of all the kit components by making aliquots according to the volume required prior freezing.
- Thaw all the kit components prior to use and keep on a cold block at all times during preparation and use.
- Affigenix's One tube Multiplex RT-qPCR reagents can be aliquoted and stored at <-10 deg C in order to maintain stability and sensitivity.

REAL TIME PCR FOR DETECTION OF COVID-19

AFFIGENIX COVID-19 TEST (ACT-1™) 3 Genes MULTIPLEX KIT protocol is designed for an in vitro detection of novel Coronavirus (2019-nCoV) RNA in respiratory specimens and sera. Our kit uses one-step real time PCR with hydrolysis probe chemistry that uses the 5′ nuclease activity of Taq DNA polymerase and enables the detection of a specific PCR product as it accumulates during PCR cycles.

AVOIDING SAMPLE CONTAMINATION

Because of the sensitivity of fluorogenic 5′ nuclease assays, special precautions must be taken to avoid false positive amplifications. Compliance with Good Laboratory Practices (GLP) is essential to minimize the risk of specimen contamination and introduction of nucleases.

The following precautionary steps are recommended:

a) Maintain separate areas for assay setup and handling of nucleic acids. Follow unidirectional workflow from reagent preparation to sample handling to PCR amplification area.
b) Maintain separate, dedicated equipment (e.g., pipettes, microcentrifuges) and supplies (e.g., microcentrifuge tubes, pipette tips) for assay setup and handling of extracted nucleic acids.
c) Wear a clean lab coat and powder- free disposable gloves (not previously worn) when setting up an experiment to avoid RNase contamination.
d) Keep reagent and reaction tubes capped or covered as much as possible.

EQUIPMENT PREPARATION

Work surfaces, pipettes, and centrifuges should be cleaned and decontaminated with cleaning products such as 5% bleach, RNase/DNase Away to minimize risk of nucleic acid contamination.

REACTION AND PLATE SET UP

Note: Keep all reagents on a cold rack during experiment set up.

1. Take and thaw all the components thoroughly and before using it, mix gently, spin down the content for 5 seconds and then test it immediately.
2. Test Controls:
   Test controls should be run concurrently with all test samples as applicable.
   - PC- Positive template control with an expected Ct value range
   - NTC- negative template control added during RT- qPCR reaction set-up
   - All clinical samples should be tested for human RNase P gene to assess specimen quality.
3. Determine the number of reactions (N) to perform for each experiment. It is necessary to make excess reaction cocktail to allow for control reactions and pipetting error.
4. Prepare the below reaction mix for each sample using the components listed below
5. For each primer/probe set, calculate the amount of each reagent to be added for each reaction mixture (N+1= No. of reactions).

Reagent	Volume per Reaction
PCR mix	8.75 µL
Multiplex mix	1.25 µL
Total reaction mix volume	10 µL
Test sample volume	10 µL

I. Mix reaction mixtures by pipetting up and down. Do not vortex.
II. Centrifuge for few secs to collect contents at the tube, and then place the tube in cold rack.
III. Set up reaction strip tubes or plates in 96-well cooler rack.
IV. Dispense 10 µL of each Reaction mix into the appropriate wells of plates/strips.
V. Prior to moving to the nucleic acid handling area, prepare the No Template Control (NTC) reactions in to Negative template control wells in the assay preparation area.
VI. Pipette 10 µL of nuclease- free water into the NTC sample wells. Securely cap NTC wells before proceeding.

VII. Cover the entire reaction plate and move the reaction plate to the specimen nucleic acid handling area.

TEMPLATE ADDITION

1. Gently vortex nucleic acid sample tubes for approximately 5 seconds.
2. After centrifugation, place extracted nucleic acid sample tubes in the cold rack.
3. Samples should be added to designated sample wells. Carefully pipette 10 µL of the sample into all the wells labelled for that sample. Keep other sample wells covered during addition. Change tips after each addition.
4. Securely cap the column to which the sample has been added to prevent cross contamination and to ensure sample tracking.
5. Change gloves often and when necessary to avoid contamination.
6. Repeat steps #3 and #4 for the remaining samples.
7. When necessary, add 10 µL of Human Specimen Control (NC) extracted sample to the NC wells securely cap wells after addition.
8. Cover the entire reaction plate and move the reaction plate to the positive template control handling area.

TEST CONTROL ADDITION

a) Pipette 10 µL of positive control to the positive control wells. Securely cap wells after addition.
b) NOTE: If using 8-tube strips, label the TAB of each strip to indicate sample position.
c) DO NOT LABEL THE TOPS OF THE REACTION TUBES!
d) Briefly centrifuge reaction tube strips for 10-15 seconds. After centrifugation return to cold rack.
e) NOTE: If using 96- well plates brief spin or centrifuge for 30 seconds at 500 x g, 4°C

EQUIPMENT PREPARATION

NOTE: Please ensure that the instruments have been installed, calibrated, checked and maintained according to the manufacturers recommendations

a) Clean and decontaminate all work surfaces, pipettes, centrifuges and other equipment prior to use using RNase Away or 10% freshly prepared bleach.
b) Turn on real time instrument and allow the block to reach optimal temperature.
c) Perform plate set up and select cycling protocol on the instrument.
d) Instrument Settings:
Run Mode: Standard
Sample Volume: **20 µL**
Passive Reference: None
Channel section: Select channels in the following sequence

Detection for	Reporter	Quencher
ORF 1ab	FAM	None
N1 gene	HEX / VIC	None
RNase P	ROX	None
E gene	Cy5	None

Thermal cycling parameters:

Parameter	Cycles	Temperature	Time
RT incubation	Hold	48°C	15 minutes
Enzyme inactivation	Hold	95°C	3 minutes
PCR amplification	40	95°C	15 seconds
		60°C	30 seconds* (*Data collection)

DATA ANALYSIS AND INTERPRETATION

Analysts should be trained and familiar with testing procedures and interpretation of results prior to performing the assay.

After completion of the run, save and analyze the data following the instrument manufacturer's instructions. Analyses should be performed separately for each target using a manual threshold setting. Thresholds should be adjusted to fall within exponential phase of the fluorescence curves and above any background signal. The procedure chosen for setting the threshold should be used consistently for every assay. Note: Set Ignore any signals from 1 to 10 cycles when necessary.

Expected performance of controls:

*Important Note: Adjust baseline of each target genes in NTC to be "undermined" before data interpretation for positive controls and clinical samples

Target	N1 (HEX)	E (Cy 5)	ORF 1ab (FAM)	RNase P (ROX)	Ct
Positive control	+	+	+	±	<38 Ct
***No template control**	-	-	-	-	-

a) NTCs should be negative and not exhibit fluorescence growth curves that cross the threshold line for the target reaction. Adjust the threshold when necessary for the target and RNase P gene when applicable. Repeated freeze-thaw and contamination in nuclease-free water will lead to nonspecific amplification. Reanalysis is recommended in such case.

b) If a false positive occurs with one or more of the primer and probe NTC reactions, sample contamination may have occurred.

c) Invalidate the run and repeat the assay with stricter adherence to the procedure guidelines.

d) PC reaction should produce a positive result with an expected Ct value for each target included in the test.

e) If expected positive reactivity is not achieved, invalidate the run and repeat the assay with stricter adherence to procedure guidelines.

f) Internal control should have Ct at or before 40 cycles for all clinical sample and NC, thus indicating the presence of sufficient nucleic acid from human RNase P gene and that the specimen is of acceptable quality.

g) Failure to detect RNase P in NC may indicate: Reagent or equipment malfunction

h) Detection of RNase P in NC but failure to detect RNase P in any of the clinical samples may indicate:
   - Improper extraction of nucleic acid from clinical materials resulting in loss of nucleic acid or carry-over of PCR inhibitors from clinical specimens
   - Absence of sufficient human cellular material in sample to enable detection

i) NC should be negative for COVID-19 target specific primer/ probe sets.
   - Contamination of nucleic acid extraction reagents may have occurred. Invalidate the run and confirm reagent integrity of nucleic acid extraction reagents prior to further testing.
   - Cross contamination of samples occurred during nucleic acid extraction procedures or assay setup. Validate the run and repeat the assay with stricter adherence to procedure guidelines.

j) When all controls meet stated requirements, a specimen is considered presumptive positive for COVID-19 in case of reaction growth curves cross the threshold line within <40 cycles for both N and E target.

k) When all controls exhibit the expected performance, a specimen is considered negative in ORF 1ab, N1 and E gene amplification curves are absent and the RNase P growth curve DOES cross the threshold line.

l) When all controls exhibit the expected performance and if all markers cycle threshold growth curve crosses the threshold line a specimen is considered positive for COVID-19. The RNase P may or may not be positive as described above, but the COVID-19 result is still valid. (according to Berlin protocol)

m) When all controls show the expected performance, but none of the genes show amplification, the result is invalid. The extracted RNA from the specimen should be re-tested. If residual RNA is not available, re-extract RNA from residual specimen and retest.

Result Interpretation:

Detection of E (Cy5)	Detection of N1 (HEX)	Detection of ORF 1ab (FAM)	Detection of RNase P(ROX)	Results
+ Ct value ≤40	+ Ct value ≤40	+ Ct value ≤40	±	COVID-19 RNA is detected
+ Ct value ≤40	-	+ Ct value ≤40	±	COVID-19 RNA is detected
-	+ Ct value ≤40	+ Ct value ≤40	±	COVID-19 RNA is detected
+ Ct value ≤40	+ Ct value ≤40	-	+ Ct value ≤40	COVID-19 RNA is presumptive positive
+ Ct value ≤40	-	-	±	COVID-19 NOT detected
-	+ Ct value ≤40	-	±	COVID-19 NOT detected
-	-	-	+ Ct value ≤40	COVID-19 NOT detected
-	-	-	-	Invalid result, repeat analysis atleast once.

*Due to differential sensitivity of the target genes (E, ORF 1ab and N1) in some cases, weak positive samples may show signal in only one of the targets. As with any diagnostic kit, results obtained in this assessment shall be interpreted in conjunction with other clinical laboratory findings.

LIMITATIONS

- Analysts should be trained and familiar with testing procedures and interpretation of results prior to performing the assay.
- A false negative result may occur if an excess of RNA template is present in the reaction. If inhibition of the RNase P control reaction is noted for a sample, extracted RNA can be tested at 2 or more dilutions (e.g., 1:10) to verify the result.

- Negative results do not exclude 2019-nCoV infection and should be used as the sole basis for treatment or other patient management decisions. Optimum specimen types and timing for peak viral levels during infections caused by 2019-nCoV have not been determined. Collection of multiple specimens (types and time points) from the same patient may be necessary to detect the virus.
- A false negative result may occur if a specimen is improperly collected, transported or handled. False negative results may also occur if amplification inhibitors are present in the specimen or if inadequate numbers of organisms are present in the specimen.

REFERENCES

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QUALITY CONTROL

Real Time PCR Detection Kit shall always be evaluated by running a positive and a negative control in each run to correctly interpret the results. Also, RNase P in each well confirms the correct performance of the technique.

Certification

- Clinically validated by ICMR National institute of Virology, Pune, India.
- CDSCO licensed to manufacture for sales and distribution.

Ordering information

Product Name	Catalogue No.	Reactions
Affigenix COVID 19 test (ACT-1™) 3 genes multiplex kit	ACT1-50	50
	ACT1-100	100
	ACT1-250	250
	ACT1-400	400

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