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What you need to know about SARS-CoV-2 qPCR detection assay

Technical note

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GenScript SARS-CoV-2 detection assay key features

- TaqMan™ qPCR assay using primers and FAM/NED/VIC probes for higher specificity;
- Targets ORF1ab gene RdRP gene, N gene and E gene in SARS-CoV-2 genome (GenBank sequences NC_045512.2);
- The assay shows positive reactivity to all four targeted genes;
- Highly sensitive and ultra-low noise;
- Full reagent set with primers, probes, reagents and positive control plasmids.

Six different assays to choose from, including CDC or WHO versions

1-step, 1-plex assay	Probe fluorescence
ORF1ab gene	FAM
RdRP gene	FAM
N gene	FAM
E gene	FAM

1-step, 2-plex assay	Probe fluorescence
ORF1ab gene +N gene (CDC guideline)	ORF1ab (NED), N(FAM)
RdRP gene + E gene (WHO guideline)	RdRP(FAM), E(VIC)

Components	Vol (μl)
2×One Step Mix	1000
One step enzyme mix	100
50×ROX Dye 1	40
50×ROX Dye 2	40
Forward Primer (10 μM)	40
Reverse Primer (10 μM)	40
Probe (10 μM)	20
RNase-free water	1000
Positive control plasmid (1 pg/μl)	100

Components	Vol (μl)
2×One Step Mix	1000
One-step enzyme mix	100
50×ROX Dye 1	40
50×ROX Dye 2	40
Primer and probe mix (N&O or R&E 10 μM)	200
RNase-free water	1000
Positive control plasmid (N&O or R&E, 0.5 pg/μl for each gene)	200

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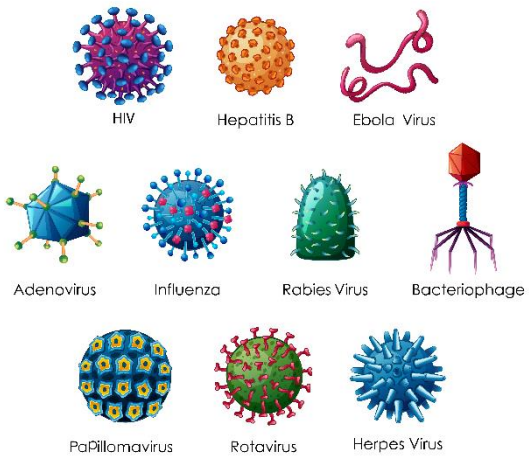
Introduction

What is a virus?



A virus is a microscopic organism that can infect all types of organisms, including animals, plants, fungi, and bacteria. It can replicate only inside the living cells. A virus can cause deadly diseases, but other times it only triggers unnoticeable reactions. The same virus can have different effects on different types of organisms, such as a plant virus that may cause the death of the plant but not be able to infect animals. Sometimes virus DNA or RNA may integrate into the host genome. The host cell then reads the inserted viral DNA and make new molecules to assemble new viruses. Virus have different shapes, such as polyhedral (Adenovirus), Spherical (Influenza), Helical (Tobacco mosaic virus) and Complex (bacteriophage).

What is coronavirus?

Coronavirus is a spherical, non-segmented RNA virus that has spike proteins protruding outside the envelope. The spike protein mediates coronavirus' entry into host cells by binding a receptor on the host cell surface.

Coronavirus has a big family that can be classified into four groups by phylogenetic clustering. They all contain large and highly conservative genomes of approximately 30 kb, including a large replicase gene. Among all four types, alpha-and beta infect mammals, and gamma infects avian species.

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Coronavirus may cause symptoms similar to a regular cold but can also cause deadly respiratory syndromes such as Severe Acute Respiratory Syndrome (SARS) in 2002, Middle East Respiratory Syndrome (2012), and COVID-19 that outbroke in Dec 2019.

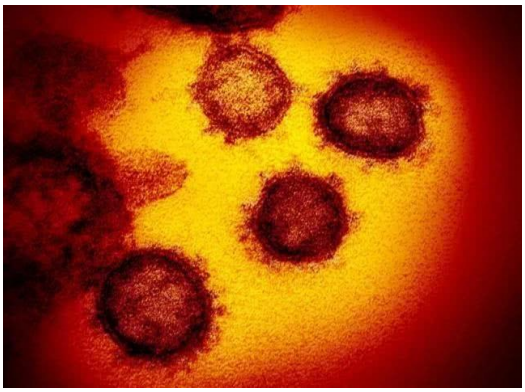
Disease name	Virus name	First emerged	Global Infected	Death	Original host
SARS	SARS-CoV	China, 2002	8,096	774	Bat
MERS	MERS-CoV	Arabia, 2012	2,470	851	Bat
COVID-19	SARS-CoV-2	China, 2019	90,870	>3,000	Unconfirmed

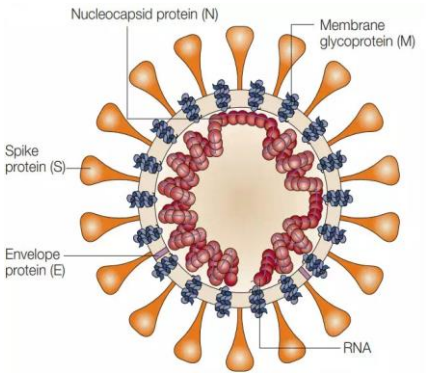
* Data up to Mar, 03, 2020

The original host of these three coronaviruses may be animals such as bats, and then transmitted to intermediate hosts such as civet or camel before infect humans. Although the original and intermediate host of COVID-19 is still unconfirmed, the virus itself has evolved to a more contagious species that have a long incubation period, causing various symptoms that are hard to identify and diagnose.

Structure of SARS-CoV-2

Coronavirus has spiked spherical envelope. National Institute of Allergy and Infectious Diseases (NIAID) scientists have identified the atomic structure of SARS-CoV-2, the virus caused COVID-19, as well as the atomic structure of its spike protein's receptor-binding domain (RBD), which could be potential vaccine targets.





Nat Rev Microbiol. 2019;17(3):181–192. Viruses. 2020;12(3):E254.

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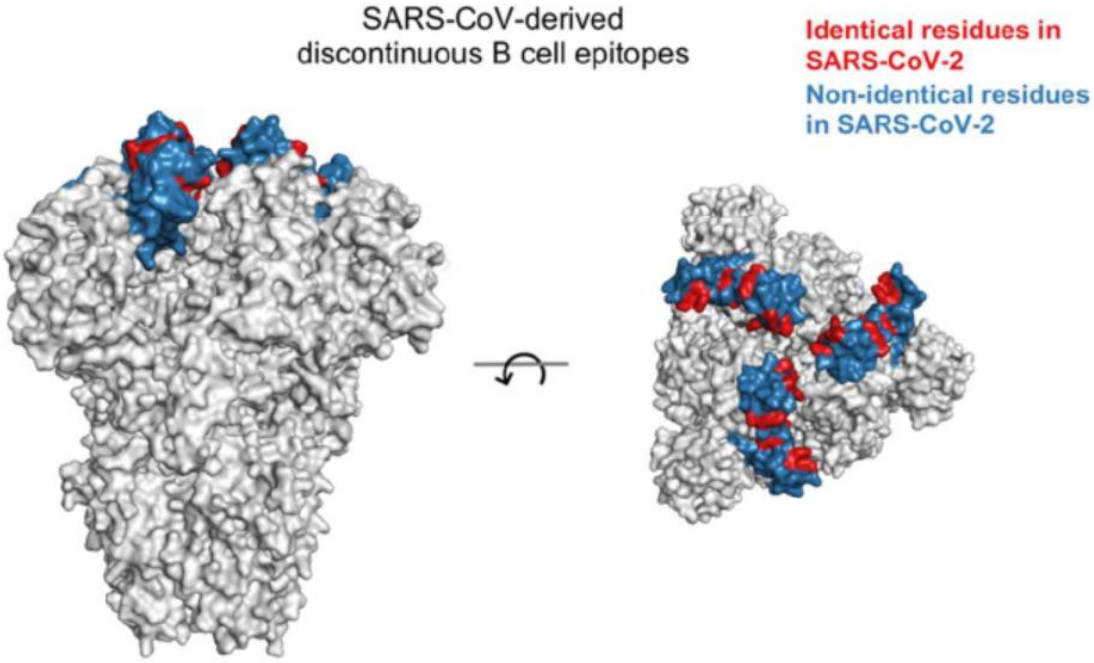

Nat Rev Microbiol. 2019 Mar; 17(3):181-192.

Treatment and diagnostics of COV-19

Since there is no specific COVID-19 treatment or vaccine available, healthcare professionals are struggling to find a provisional treatment plan that may have better survival rates. Some effective treatments include concomitant drugs of lopinavir/ritonavir with umifenovir and interferon, suppression of cytokine storm, oxygen therapy, and prevention of secondary infection. Doctors suggest starting the treatment as soon as possible, preferably within 72 hours of showing symptoms.

However, COV-19 has a longer incubation time than SARS and its symptoms resemble influenza. It is very hard to distinguish it from normal flu without the help of certain diagnostic methods. Moreover, the same set of diagnostic methods are also required to monitor disease progression or treatment effectiveness. The most widely used diagnostic methods of COVID-19 combines several tests including but not limited to:

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1) Blood test shows elevated levels of alanine aminotransferase (ALT), lactate dehydrogenase (LDH), muscle enzymes, myoglobin or troponin.

2) Chest CT scan shows a wide range of abnormalities in focused areas of ground-glass opacity (GGO) or excess fluid in one lung to diffuse buildup in both lungs.

3) SARS-CoV-2 nucleic acid test by next-generation sequence(NGS), quantitative real-time PCR, loop-mediated isothermal amplification(LAMP) or CRISPR

4) SARS-CoV-2 protein detection by colloidal gold immunochromatography or enzyme-linked immunosorbent assay (ELISA)

COVID-19 nucleic acid test

On Jan 12, 2020, SARS-CoV-2 genome sequence was published on virological.org. Several other labs soon posted similar sequences from patient samples worldwide. Up to Feb 24, 2020, 129 full-length genomes are posted on the GISAID and NCBI Genbank (virological.org), with limited sequence variations. Although 60-90% part of SARS-CoV-2 genome sequence is highly conservative and resembles other coronaviruses, scientists have identified a highly specific region that could serve as target genes for nucleic acid tests. ORF1ab (RdRP is part of it), E and N gene in this region share no common sequences with all other known coronaviruses such as SARS-CoV, MERS-CoV. WHO and CDC later published qPCR protocols against ORF1ab +N gene, or RdRP +E gene as guidelines for SARS-CoV-2 detection. It is now the most widely used nucleic acid test in COV-19 diagnosis.

This qPCR assay is based on Taqman™ qPCR technology. The primers and probes in this assay are designed based on the gene sequences of the target virus protein, including ORF1ab, N, RdRP, and E gene in SARSS-CoV-2. The single or dual-labeled probes can hybridize specifically with a part of the gene sequences. During PCR, the 5' fluorophore from the probe will be digested by Taq polymerase via its 5'-3' exonuclease activity and released from 3' quencher group, and its

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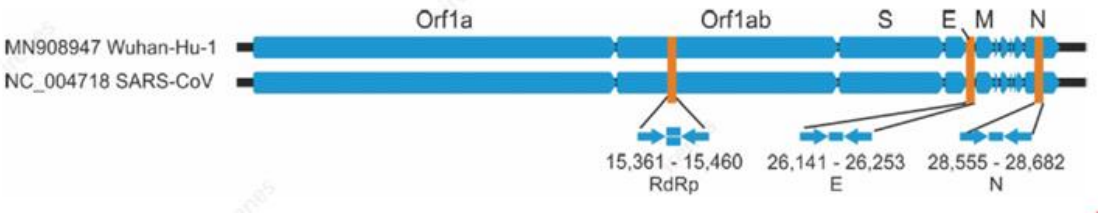
fluorescence can then be measured quantitatively by qPCR instrument. Since qPCR detects the virus via its unique sequences, the assay is highly specific to SARS-CoV-2.



Euro Surveill. 2020; 25

Why some qPCR tests are inconsistent?

During the incubation period, the COVID-19 virus is still highly contagious. Pre-symptomatic or asymptomatic patients can still spread the disease. Thus, people wish they could rely on highly specific nucleic acid tests as qPCR to screen out virus carriers so we can provide immediate treatment to them or prevent the disease from further spreading. Unfortunately, some qPCR kits were reported not able to detect infected patients or even produce consistent results.

Detection results by six NMPA (National Medical Products Administration) approved kits were compared together using the throat swab of the same patient collected at different disease progression stages (Jan-27, Jan-31, Feb-01, 2020). Interestingly, the results vary a lot among different kits or even the same kit with samples collected at different times.

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Kit	Sample ID	Target gene and result	
		N	ORF1ab
A	20200127312001	++	-
	20200127312001	++	-
	20200127312001	++	++
B	20200127312001	++	++
	20200127312001	++	-
	20200127312001	++	-
C	20200127312001	++	++
	20200127312001	+	+
	20200127312001	+	++
D	20200127312001	-	++
	20200127312001	-	++
	20200127312001	-	++
E	20200127312001	+	-
	20200127312001	+	++
	20200127312001	+	++
F	20200127312001	++	++
	20200127312001	++	++
	20200127312001	++	++

This inconsistency may not only due to the reagents or probes in the detection kits. In fact, various factors may affect qPCR assay results, such as:

1. Quality of the sample

- How the samples are collected and prepared
- How RNA are extracted from different types of samples, which may result in RNA input variations
- How samples are stored and shipped
- How many copies of the virus in the sample

There are many types of patient samples eligible for qPCT test. However, various samples may reflect the virus infection quite differently. SARS-CoV-2 may attack various tissues case by case and shows various abundances all over the human body. Samples were also collected at different

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stages of disease progression. Thus we may not always get detectable viruses in the sample, especially at the very early stage of infection. Most of the time, healthcare professionals may collect these samples for SARS-CoV-2 detection:

- Upper respiratory specimen: nasal swab, throat swab, nasopharyngeal extract.
- Lower respiratory specimen: deep sputum, respiratory tract extract, bronchial lavage fluid, alveolar lavage fluid, lung tissue biopsy.
- Blood samples: collect the blood with anticoagulant 7 days after symptoms occur.
- Eye conjunctiva: some patients may show infection in their eyes; in this case, eye conjunctiva swab can also be collected for tests.
- Stool specimen: if patient have diarrhea, stool specimen should also be collected for the test.

The copy number or concentration of virus RNA in these samples, and the sample texture or processing methods vary, resulting in a wide range of RNA input for qPCR assay.

2. Sensitivity and specificity of the detection assay due to reagents

- The efficiency of reverse transcriptase and DNA polymerase.
- The abundance and specificity of the probe.
- The conjugate efficiency of the fluorophore with the probe.
- The background fluorescence blocked by the quencher.

All these can affect the limit of detection, as well as the specificity and consistency of qPCR assay. Due to the tremendous needs of IVD assay during COV-19 outbreak, NMPA in China expedite the approval process of SARS-CoV-2 qPCR detection kit to allow more diagnostic labs to develop their own kits. However, with the limited assay development time, not all kits were optimized and verified enough to offer a clear definition of the limit of detection (LoD), or allocated material production by reliable suppliers to ensure consistent performance.

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3. Experimental procedure

- Is the protocol strictly followed?
- Has the protocol been validated for different sample inputs or instruments?
- Is the technician experienced and familiar with qPCR assay and the lab's standard operating procedure?

For example, to use the test for COVID-19 diagnosis, samples from patients, such as lavage fluid, need to be collected and preserved well in a sealed specimen tube, transfer to biosafety level 1 lab to extract RNA, then use qPCR assay to read the real-time fluorescent signal during RNA reverse transcription. The result will be analyzed based on the # of PCR cycles before the fluorescent signal passes the threshold (Ct). A reliable qPCR assay usually has Ct>40 for negative controls (NTC Ct>40), and positive control Ct value around 26. The results are considered positive if the Ct is 30 -37. During this process, sample type, volume, total input RNA after extraction should be clearly defined and strictly followed. Also, the PCR program, the instrument, and the fluorescent dye will also affect the Ct value.

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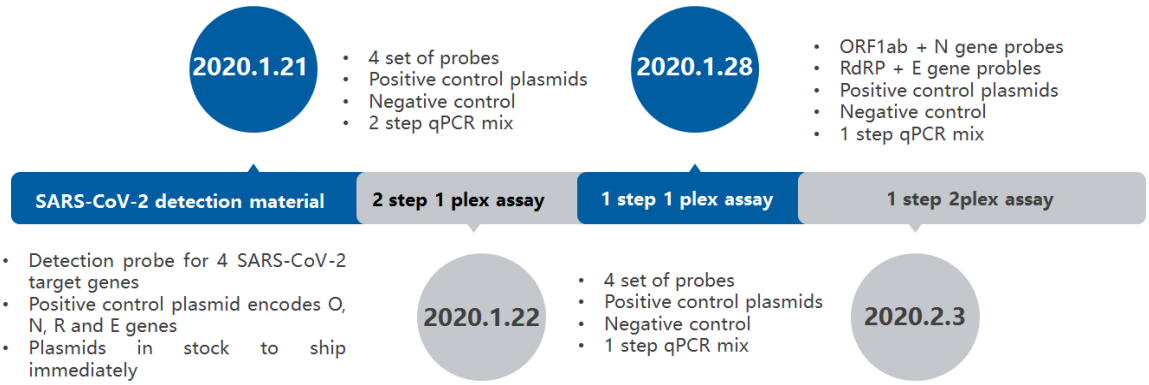
GenScript SARS-CoV-2 detection assay

GenScript is the world leader in biotechnology reagent services and biologics. We work with the pharmaceutical industry by providing custom gene and peptide synthesis, protein expression, and engineering, custom antibody development and engineering, in vitro/in vivo pharmacology as well as a variety of catalog products. In response to the outbreak of COVID-19, our R&D team developed qPCR TaqmanTM detection assay reagent sets that serve as reliable starting materials to develop COVID-19 IVD test. We also have a large capacity to fulfill the needs worldwide and we will prioritize the production of any reagents related to SARS-CoV-2 study.

Timeline of SARS-CoV-2 detection assay development



GenScript SARS-CoV-2 detection assay provides a method to detect the virus even at a very low level, leading to the possibility of virus detection at a very early stage. The 1-step 1-plex or 2-plex qPCR assay typically takes 1 hour after RNA extraction and is fully compatible with high-throughput liquid handlers.

GenScript offers four different types of 1-step 1-plex assay, targeting 4 different SARS-CoV-2 genes, and two different types of 1-step 2-plex assay, targeting ORF1ab+N gene or RdRP+E gene. All 6 assays are sensitive enough to detect 0.001 pg of control plasmid in 20ul reaction. Also, since all primers and probes were purified at our highest HPLC+ grade to ensure >90% purity and <0.05%

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cross-contamination, leading to ultra-flow false positive rate. The negative control Ct value is >40-45.

The assay can help scientists develop an IVD assay to help quickly confirm a diagnosis or screen the suspects along with other diagnostic methods.

Since the virus can cause various symptoms, and is highly contagious, identifying infected individuals not only help to provide the corresponding medical treatment but also significantly control the spread of the disease. On the other hand, researchers may study the mechanism of the disease or efficacy of the treatments by using the assay to quantitatively monitoring the virus level. GenScript will work hard to develop reagents to help global community fight against COVID-19.

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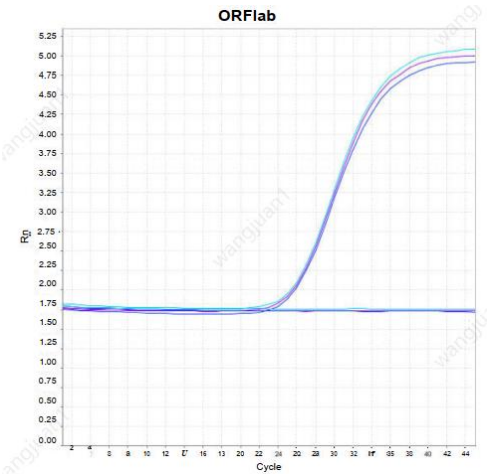
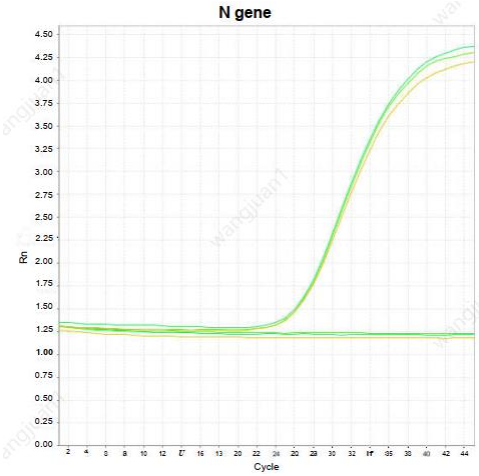
1-step 1-plex assay: your choice of 4 target genes

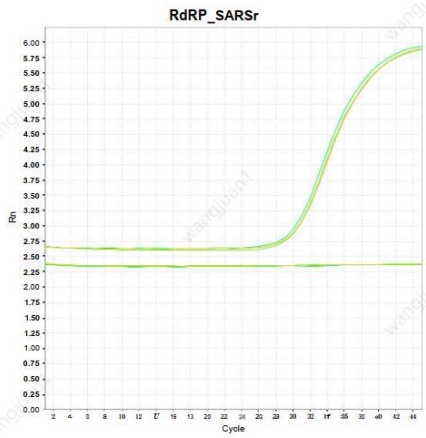
Components	Vol (μl)
2×One Step Mix	1000
One step enzyme mix	100
50×ROX Dye 1	40
50×ROX Dye 2	40
Forward Primer (10 μM)	40
Reverse Primer (10 μM)	40
Probe (10 μM)	20
RNase-free water	1000
Positive control plasmid (1 pg/μl)	100



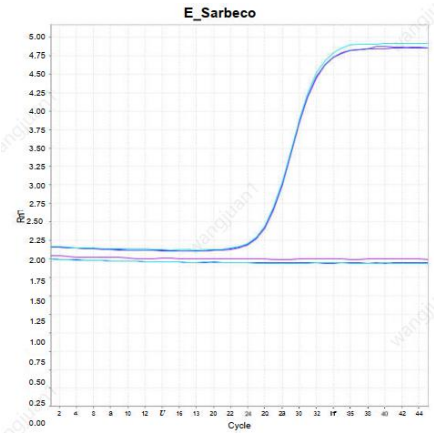
- O gene positive ctrl Ct: 25.9
- NTC: N.A.



- N gene positive ctrl Ct: 26.4
- NTC: N.A.



- R gene positive ctrl Ct: 30.3
- NTC: N.A.



- E gene positive ctrl Ct: 26.0
- NTC: N.A.

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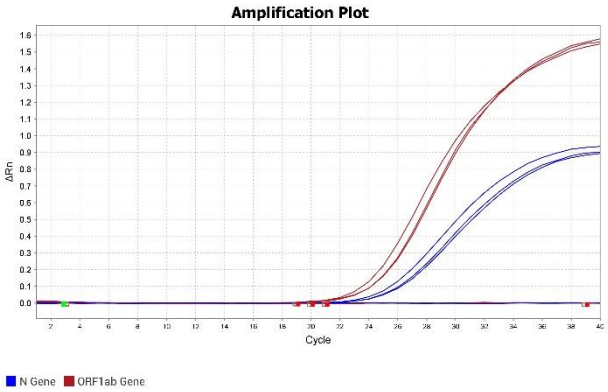
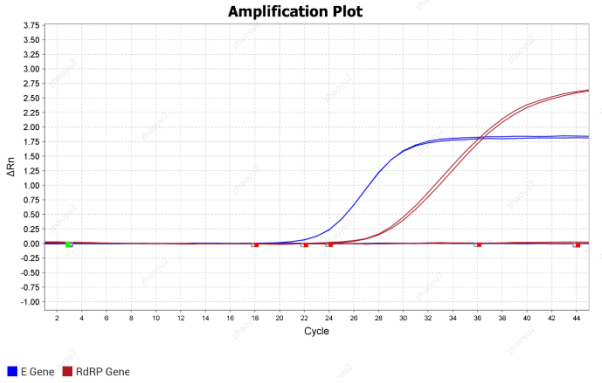
1-step 2-plex assay: your choice of ORF1ab+N or RdRP+E gene pair

Components	Vol (µl)
2×One Step Mix	1000
One-step enzyme mix	100
50×ROX Dye 1	40
50×ROX Dye 2	40
Primer and probe mix (O&N or R&E 10 µM)	200
RNase-free water	1000
Positive control plasmid (O&N or R&E, 0.5 pg/µl for each gene)	200



- ORF 1ab gene positive ctrl Ct: 24
- N gene positive ctrl Ct: 25
- NTC: N.A.



- RdRP gene positive ctrl Ct: 27
- E gene positive ctrl Ct: 23
- NTC: N.A.

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