

Real-time RT-PCR for diagnosing 2019-nCoV

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1 Introduction

This video takes you through a polymerase chain reaction (PCR) method for diagnosing the 2019-nCoV virus from patient samples. It utilises the virus' nucleic acid, therefore this technique can be used for detecting 2019-nCoV in animals as well. The information provided in this video, such as the primer sequences, kits used and Thermocycler conditions, are from Huang et al. paper published in January 2020:

Huang et al. Clinical features of patients infected with 2019 novel coronavirus in Wuhan, China. *Lancet* 2020; **6736**: 20: 1-10

[https://www.thelancet.com/journals/lancet/article/PIIS0140-6736\(20\)30183-5/fulltext](https://www.thelancet.com/journals/lancet/article/PIIS0140-6736(20)30183-5/fulltext)

There are other papers too, including the one linked below, which uses primers aimed at a different gene:

Chan et al. A familial cluster of pneumonia associated with the 2019 novel coronavirus indicating person-to-person transmission: a study of a family cluster. *Lancet* 2020; **6736**: 20: 1-10 [https://www.thelancet.com/journals/lancet/article/PIIS0140-6736\(20\)30154-9/fulltext](https://www.thelancet.com/journals/lancet/article/PIIS0140-6736(20)30154-9/fulltext)

1.1 Other sources of information

The Global Health Network has set up a 2019-nCoV knowledge hub for guidance:

<https://coronavirus.tghn.org/>

The WHO have produced a short course on respiratory viruses, including 2019-nCoV:
<https://openwho.org/courses/introduction-to-ncov>

2 Protocol

2.1 Important information

2019-nCoV is a biological safety level 3 pathogen, as there is evidence that it can be transmitted from human to human, and you therefore **must** have biosafety level three facilities to isolate the RNA from the patient samples and a suitable clean chain of laboratories to prevent contamination.

The extraction of RNA must be done in a class I or class II microbiological safety cabinet (MSC) within a BSL3 facility to protect yourself from contamination. The eluted RNA can then be removed from the BSL3 laboratory and processed in the molecular biology area of your laboratory (e.g. Section 2.2.2 is the first part of this protocol that can be done in a BSL2 laboratory).

2.2 Preparation of viral RNA

Before you can do PCR on your samples, you will need to extract the RNA from them first. The 2019-nCoV virus is an enveloped single stranded RNA virus. It is the envelope gene that we will be targeting in the PCR. Plasma is used for this, but it is important to also screen for other viruses, common in your country or area, as well as obtaining as much clinical data from the patient and contacts, to ensure that you have as much information as possible to make the correct diagnosis.

2.2.1 Extraction of RNA

Many viral RNA extraction kits are available, but the kit mentioned in the paper and this video is the Direct-zol RNA MiniPrep kit (R2050) Zymo research, Irvine, CA, USA. You then need to follow the extraction kit instructions, and a link to the kit can be found here:

https://www.bioscience.co.uk/userfiles/pdf/R2050_R2051_R2052_R2053_Direct-zol_RNA_MiniPrep_ver_1_1_3.pdf

The protocol is in two parts, sample preparation, where you add the Trizol or other similar guanidinium thiocyanate-phenol-chloroform reagents to the sample that will disrupt the viral capsid and envelope to release the nucleic acid and then the purification step, where you wash the RNA on a column using ethanol-based washing buffers and centrifugation, before eluting it into 50 µL nuclease free water. The purified RNA can then be used immediately or stored at -70°C.

2.2.1.1 Sample preparation

- Prepare buffers by adding ethanol to the **Direct-zol™ RNA PreWash** and **RNA Wash Buffer** concentrates (check the instructions on your kit for volumes)
- Add 80 µL of your patient's or contact's plasma sample to 240 µL **Trizol LS** (10296028, Thermo Fisher Scientific, Carlsbad, CA, USA) or **TRI Reagent®** and mix thoroughly for 5 minutes. *NB. Trizol is the brand name of a guanidinium thiocyanate buffer and is used as a nucleic acid protector, to improve the yield of RNA.*
- To remove particulate debris, centrifuge and transfer the supernatant to an RNase-free tube. When centrifuging, carefully wipe down the tubes with disinfectant before placing them into enclosed microcentrifuge bucket to contain any aerosols produced. Wipe down the outside of the bucket and your gloves with disinfectant (following your local safety rules regarding disinfection) and then remove it from the microbiological safety cabinet.

2.2.1.2 Sample Purification

All centrifugation steps should be performed at 10,000-16,000 x *g* for 30 seconds. You will need a microcentrifuge bucket that will contain any aerosols produced. Loading and unloading the bucket should be undertaken in the MSC (see the video for more details).

1. Add an equal volume ethanol (95-100%) to a sample lysed in **TRI Reagent®** or similar and mix thoroughly
2. Transfer the mixture into a **Zymo-Spin™ IIC Column** which is placed in a **Collection Tube** and centrifuge. Transfer the column into a **new** collection tube and discard the flowthrough

NB an extra recommended step: **DNase I** treatment (in-column)

- Add 400 µL **RNA Wash Buffer** to the column and centrifuge.
 - In an RNase-free tube, add 5 µL **DNase I** (6 U/µL)*, 75 µL **DNA Digestion Buffer** and mix. Add the mix directly to the column matrix
 - Incubate at room temperature (20-30°C) for 15 minutes. Proceed to step 3.
3. Add 400 µL **Direct-zol™ RNA PreWash** to the column and centrifuge. Discard the flow-through and repeat this step
 4. Add 700 µL **RNA Wash Buffer** to the column and centrifuge for 2 minutes to ensure complete removal of the wash buffer. Transfer the column carefully into an RNase-free tube
 5. To elute RNA, add 50 µL of **DNase/RNase-Free Water** directly to the column matrix and centrifuge. Alternatively, for highly concentrated RNA use ≥25 µL elution

The eluted RNA can be used immediately or stored at ≤-70°C. Ensure that you keep track of each of your samples by labelling them clearly.

2.2.2 Checking your RNA (quality and concentration)

As good practice, it is worth checking that your extraction method worked and you have at least some RNA. There are a number of ways to do this

2.2.2.1 Nanodrop

For this, you will need a Nanodrop spectrophotometer and a computer/laptop with the Nanodrop programme installed. The Nanodrop will also give you the purity of the DNA, using the 260/280 ratio. Please click on the link for a protocol for using the Nanodrop:

Protocol for the Nanodrop 2000/2000c Spectrophotometer:

https://www.mlz-garching.de/files/nanodrop_2000_user_manual.pdf

YouTube video protocol: <https://www.youtube.com/watch?v=FiGZnNs2xXY>

Or, follow the link below to the manufacturers website:

<https://www.thermofisher.com/uk/en/home/industrial/spectroscopy-elemental-isotope-analysis/molecular-spectroscopy/ultraviolet-visible-visible-spectrophotometry-uv-vis-vis/uv-vis-vis-instruments/nanodrop-microvolume-spectrophotometers.html>

DNA purity calculated using the 260/280 ratio is explained below -

<https://www.nhm.ac.uk/content/dam/nhmwww/our-science/dpts-facilities-staff/Coreresearchlabs/nanodrop.pdf>

2.2.2.2 Qubit

For this you will need a Qubit fluorometer reader and one of the assay kits.

YouTube protocol video - <https://www.youtube.com/watch?v=RRKZN--7jqg>

Protocol for the Qubit dsDNA high sensitivity (HS) assay kit -

https://www.thermofisher.com/document-connect/document-connect.html?url=https%3A%2F%2Fassets.thermofisher.com%2FTFS-Assets%2FMSG%2Fmanuals%2FQubit_dsDNA_HS_Assay_UG.pdf&title=VXNiciBHdWlkZTogUXViaXQgZHNETkEgSFMgQXNzYXkgS2I0cw==

Protocol for the Qubit RNA high sensitivity (HS) assay kit -

https://assets.thermofisher.com/TFS-Assets/MSG/manuals/Qubit_RNA_HS_Assay_UG.pdf

Link to the Qubit web page -

<https://www.thermofisher.com/uk/en/home/industrial/spectroscopy-elemental-isotope-analysis/molecular-spectroscopy/fluorometers/qubit.html>

2.2.2.3 Tapestation

The Agilent TapeStation system is an automated electrophoresis tool for DNA and RNA sample quality control. The machine can analyse the size, concentration and integrity of your sample.

Click on this link for a quick guide –

https://www.agilent.com/cs/library/usermanuals/Public/ScreenTape_gDNA_QG.pdf

YouTube protocol tutorial - <https://www.youtube.com/watch?v=XI74ZZghR5o>

2.3 Reverse transcriptase PCR

2.3.1 PCR reagents

Polymerase Chain Reaction (PCR) requires DNA. As the method in section 2.2.1 extracts viral RNA, we need to use reverse transcriptase to create complimentary DNA (or cDNA) strands from the extracted RNA.

In this protocol, we are using One-Step real-time RT-PCR, where the cDNA is synthesised by a reverse transcriptase in the same tube as the PCR reaction. Compared to two-step real-time RT-PCR (in which the cDNA is synthesised separately and then added into the PCR reaction) it is simple and rapid, with fewer pipetting steps (reducing possible errors and contamination).

You can click on the following link to watch a YouTube video explaining real-time RT-PCR:
https://www.youtube.com/watch?v=DH7o9Df5_50

2.3.2 Primers and probe

To undertake this PCR, you will need primers and a probe specific to the organism that you are trying to detect, in this case the 2019-nCoV. There are a number of companies that you can buy primers from.

Usually primers will be delivered to you as a lyophilised powder (although often this powder is barely visible to the human eye, it is spread around the walls and lid of the tube. To use your primers, they will need to be reconstituted in nuclease free water. You should receive a data sheet with your primers telling you what volume is needed to make a stock solution of (usually) 100 μ M (although this depends on the company, you might have to do some calculations to work it out). Ensure you have thoroughly mixed the water with the powder and before opening your tube, spin it down. It is advisable to then aliquot some of this stock solution into microtubes of working solution of 10 μ M and freeze them at -20°C, to minimise freeze thawing and contamination of your main stock.

Probes also usually come lyophilised and need reconstituting in the same way. This may differ depending on the manufacturer, so again, please check their instructions.

Primer and probe sequences for the 2019-nCoV virus, which targets the envelope protein gene (E) have been published in the paper:

Forward primer 5'-TCAGAATGCCAATCTCCCAAC-3'

Reverse primer 5'-AAAGGTCCACCCGATACATTGA-3'

Probe 5'-CY5-CTAGTTACTAGCCATCCTTACTGC-3'-BHQ1

Other primer sequences have also been published to detect coronaviruses. Please refer to the literature.

Note that your real time machine must have the channel filter to detect the fluorescence from the CY5 dye. If your machine doesn't have this filter, you can choose any other dye that is detected by your machine (e.g. FAM, HEX, JOE). The BHQ1 quencher also can be modified according to the dye you choose. Usually, the manufacturer website will provide a list of available dye-quencher combinations for you.

An example can be found here:

<https://www.idtdna.com/pages/education/decoded/article/qpcr-probes-selecting-the-best-reporter-dye-and-quencher>

However, if you do not know your primer sequences (for example, if you are designing a PCR to identify a new organism, or a different target gene) there are websites that explain primer design:

University of Glasgow - https://www.youtube.com/watch?v=c-f1H07D_70

The links below are to some websites and publications that explain how to design primers:

- Genome Compiler - <http://www.genomecompiler.com/tips-for-efficient-primer-design/>
- AddGene - <https://www.addgene.org/protocols/primer-design/>
- ThermoFisher - <https://www.thermofisher.com/uk/en/home/life-science/oligonucleotides-primers-probes-genes/custom-dna-oligos/oligo-design-tools.html>
- Revisiting primer design - <https://www.ncbi.nlm.nih.gov/pmc/articles/PMC5702850/>

There are also online tools that can help you design primers (some are free, others are not):

- SciTools - <https://www.idtdna.com/pages/education/decoded/article/designing-pcr-primers-and-probes>
- Eurofins genomics - <https://www.eurofinsgenomics.eu/en/dna-rna-oligonucleotides/oligo-tools/primer-design-tools/>
- NCBI - <https://www.ncbi.nlm.nih.gov/tools/primer-blast/>
- Primer3 - <http://primer3.ut.ee/>

2.3.3 Positive controls

You are unlikely to have a positive control for this real-time RT-PCR, unless you already have RNA from a confirmed positive 2019-nCoV case. According to the WHO (<https://www.who.int/publications-detail/laboratory-testing-for-2019-novel-coronavirus-in-suspected-human-cases-20200117>) to verify false-positives, you are advised to test your samples for other coronavirus using MERS-CoV, SARS-CoV and/or PAN-CoV protocols.

To verify the presence of true-positives you must sequence your PCR products and check if the sequence you obtained correspond to 2019-nCoV (GenBank accession number: MN938384 and MN975262). Once you have identified positive samples, you are advised to

clone them into plasmid vectors to make a stock of positive control. You can use your cloned positive control to validate this reaction in your lab.

2.3.4 Negative controls

You should always run negative controls in your RT-PCR, to ensure that no contamination has entered during the protocol. Ideally, you should have a negative control from the beginning, i.e. throughout the extraction steps too (instead of a serum sample, you add Trizol and process 80 µL nuclease-free water alongside your other samples). You could also run a no template control (NTC) or blank, so instead of adding 5 µL RNA into the PCR reaction tube, add 5 µL nuclease free water.

2.3.5 Reagent volumes for PCR reaction

We are using the AgPath-ID™ One-Step RT-PCR reagents (AM1005, Thermo Fisher Scientific), as these were used in the paper we are referencing. These reagents are designed for sensitive, robust amplification of RNA targets using a rapid single-tube TaqMan™ real-time reverse transcription PCR (RT-PCR) strategy. The reactions are assembled in a single tube, minimizing sample handling errors and expediting set-up time. Once assembled, results are available in approximately one hour.

The 25X RT-PCR Enzyme Mix included in the kit contains highly efficient ArrayScript™ Reverse Transcriptase, a mutant MMLV RT that produces high cDNA yields, and AmpliTaq Gold™ polymerase, the preferred hot-start DNA polymerase for specific target amplification. A hot-start DNA polymerase increases the specificity of the reaction because the enzyme is activated at high temperatures, for instance after the denaturation step (~95°C), avoiding undesirable amplification at room temperature.

The 2X RT-PCR Buffer has been optimized for efficient, robust reverse transcription and PCR includes the passive reference dye, ROX™ Dye, for quantitative fluorescent signal normalization. A Detection Enhancer is also provided as an optional reagent for amplification of templates with a high GC content or persistent secondary structure. This buffer will provide a perfect environment for the reaction to happen, like optimised concentrations of DNA polymerase cofactor ions (Mg⁺⁺, Mn⁺⁺ or equivalent), the reverse transcriptase and DNA polymerase substrates aka dNTPs, appropriated pH and other ideal conditions for the enzymatic reactions.

NB When you are working out the quantity of each reagent that you need, it is advisable to prepare 5-10% extra master mix, as you will lose small amounts when moving reagents between tubes.

NB If you are using a different polymerase, read the manufacturer's instructions to identify the optimum volumes for each reagent.

NB If you have to change the volume of any of the reagents in the master mix, recalculate and then make the volume up with nuclease-free water.

Component		Volume	Volume for 12 x (9 samples, 1 negative control + 2 as 'extra') reactions
Real-time RT-PCR master mix	Nuclease-free water	3.5 µL	42 µL
	2X RT-PCR Buffer	12.5 µL	150 µL
	Forward primer (from 10 µM working stock)	1.25 µL	15 µL
	Reverse primer (from 10 µM working stock)	1.25 µL	15 µL
	Probe	0.5 µL	6 µL
	25X RT-PCR enzyme Mix	1 µL	12 µL
RNA sample (in Nuclease-free water)		5 µL	<i>Do not add samples to master mix</i>
Total volume per reaction		25 µL	25 µL x 12

2.3.6 Setting up the Thermocycler

2.3.6.1 Thermocycler conditions

Your Real-time PCR machine may be different from the one used in the video, so setting up your run may be different.

The RT-PCR cycle conditions for 2019-nCoV, as stated in the paper are:

- 50°C for 15 minutes
- 95°C for 3 minutes
- 45 cycles of 95°C for 15 seconds and 60°C for 30 seconds

NB It may require some optimisation for your own machine and reagents, please refer to the manufacturer's instructions to ensure optimum cycle conditions.

3 Interpreting results

For this diagnostic RT-PCR, we are not quantifying these samples. It is a qualitative protocol, i.e. we are just looking to see whether the DNA is present (therefore, whether this patient has 2019-nCoV) or not. We are looking to see whether the lines representing the samples are above the threshold.

The threshold line is defined as the level of detection or the point at which a reaction reaches a fluorescent intensity above background levels (i.e. your negative controls). To validate this

PCR for the first time (if you do not already have a 2019-nCoV positive control), you should sequence your positive samples.

Thermo Fisher have produced a series of YouTube videos, which help to explain real-time PCR and the results: <https://www.thermofisher.com/uk/en/home/life-science/pcr/real-time-pcr/real-time-pcr-learning-center/real-time-pcr-basics/ask-taqman-video-series.html>