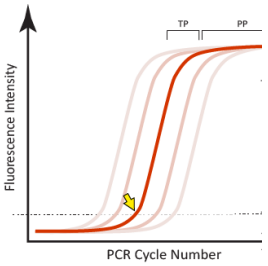
Feb 04, 2020 Version 3

Novel coronavirus (2019-nCoV) real-time RT-PCR N gene 2020 (Wuhan-N; 2019-nCoV-related test) -**NOT RECOMMENDED** V.3



DOI
https://dx.doi.org/10.17504/protocols.io.bb5piq5n

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DOI: https://dx.doi.org/10.17504/protocols.io.bb5piq5n

Protocol Citation: Judy A. Northill, Ian Mackay 2020. Novel coronavirus (2019-nCoV) real-time RT-PCR N gene 2020 (Wuhan-N; 2019-nCoV-related test) -NOT RECOMMENDED. **protocols.io** https://dx.doi.org/10.17504/protocols.io.bb5piq5n

Protocol status: In development
We are still developing and optimizing this protocol, though have found the sensitivity is lower than expected.

Created: February 04, 2020

Last Modified: February 04, 2020

Protocol Integer ID: 32655

Keywords: CoV, coronavirus, Wuhan, Real-time, RT-PCR, PCR, virus, China, 2019-nCoV, pneumonia, seafood market, WSMPV, detecting wuhan virus, wuhan virus, novel coronavirus, betacoronavirus, sars virus, final name for this virus, sequence mn908947, nucleocapsid, orf1ab assay, type virus, betacov, pcr

Abstract

- **NOT RECOMMENDED**
- The sensitivity of the assay has been found to be lower than expected and we no longer recommend it be used.
- We do recommend the ORF1ab assay (**Novel coronavirus (2019-nCoV) real-time RT-PCR ORF1ab 2020**) or the E gene assay by Corman et al. (**Protocol v2-1**)

- A real-time RT-PCR to designed to detect the "novel Wuhan" betacoronavirus. Based on sequence MN908947 made available by Professor Yong-Zhen Zhang, Fudan University, Shanghai, China.
- The target region encodes the nucleocapsid (N).
- Not tested on wild-type virus (as of 25Jan2020), it is expected to be capable of detecting Wuhan virus, bat-like SARS and SARS virus.
- Limit of detection not yet determined.
- A single 1 mismatch at probe-binding site identified with the *BetaCoV/USA/CA1/2020|EPI_ISL_406034* variant of 2019-nCoV (as of 29JAN2020).
- Probe is in the 3'-5' (reverse complement) direction.

Notes:
1. Assay is optimised (as of 24Jan2020).
2. A final name for this virus has not been decided (as of 25Jan2020).

Guidelines

- If using a different brand or model of real-time thermocycler, check the concentration of ROX is adequate.
- Method assumes the user is familiar with the thermocycler and software used to run the protocol.

Materials

STEP MATERIALS

SuperScript™ III Platinum™ One-Step qRT-PCR Kit **Life Technologies Catalog #**11732088

Protocol materials

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Mix

1 **Oligonucleotides**

	Oligo Name	Sequence 5'-3'	Location based on NC_045512*
	Wuhan-TM2020For	TCGTGCTACAACTTCCTCAAG	28648-28668
	Wuhan-TM2020Probe	6FAM-CCGCCTCTGCTCCCTTCTGC-BHQ1	28714-28695
	Wuhan-TM2020Rev	CTGCCWGGAGTTGAATTTCTTG	28780-28759

*GenBank accession NC_045512 Wuhan seafood market pneumonia virus isolate Wuhan-Hu-1

2 **Reagents**

SuperScript™ III Platinum™ One-Step qRT-PCR Kit **Life Technologies Catalog #**11732088

3 **Synthetic controls**

Synthetic controls are produced using the **binary synthetic template oligonucleotide positive control for in-house diagnostic real-time RT-PCR method.**

The oligonucleotide sequences required to make controls for this assay are:
Probe control:

AAAATAATACGACTCACTATAGGGTGAAGAGAATCCACAAGGAATTGAACCGCCTCTGCTC
CCTTCTGCACAGTGTTCAGCAGGTCCTGTTGAAAA
Primer control:
AAAATAATACGACTCACTATAGGGTCGTGCTACAACTTCCTCAAGATGATCTGGCACGGGAC
CCTCCAACAAGAAATTCAACTCCAGGCAGAAAA

4 **Reaction Set-up**

- Assay has been designed to be used on both a Rotor-Gene 6000 / Rotor-Gene Q 5-plex using 100-place rotor discs and an ABI 7500 Fast real-time machine.
- Total reaction volume is 20µL.
- Prepare sufficient for number of reaction plus a 'dead volume' usually 2 extra. Adjust as necessary if using a robotic dispenser.

	Reagent	Volume (ul) X1	Final reaction concentration
	Nuclease free water	4.41	
	Wuhan-TM2020F (200uM)	0.05	500nM
	Wuhan-TM2020R (200uM)	0.09	900nM
	Wuhan-TM2020Probe (100uM)	0.01	50nM
	2 X Reaction mix*	10	1X
	Superscript III/Platinum Taq enzyme mix*	0.4	
	ROX reference dye (25uM)*	0.04	50nM
	TOTAL VOLUME	**15**	

*Superscript®III Platinum® One-Step qRT-PCR kit

Dispense 15µl to each reaction well.
Add 5µl of template, extracted RNA, controls or NTC (nuclease-free water).
Total reaction volume is 20µl.

Amplification

5 **PCR amplification**

	1 cycle	40 cycles
	50° C 5min	95° C 3 seconds
	95° C 2min	60° C 30 seconds*

*Florescence acquisition step

Result Analysis

6 The definition used for a satisfactory positive result from a real-time fluorogenic PCR should include each of the following:

1. A sigmoidal curve – the trace travels horizontally, curves upward, continues in an exponential rise and followed by a curve towards a horizontal plateau phase
2. A suitable level of fluorescence intensity as measured in comparison to a positive control (y-axis)
3. A defined threshold (C_T) value which the fluorescent curve has clearly exceeded (Fig.1 arrow) and which sits early in the log-linear phase and is <40 cycles
4. A flat or non-sigmoidal curve or a curve that crosses the threshold with a C_T value >40 cycles is considered a negative result
5. NTCs should not produce a curve

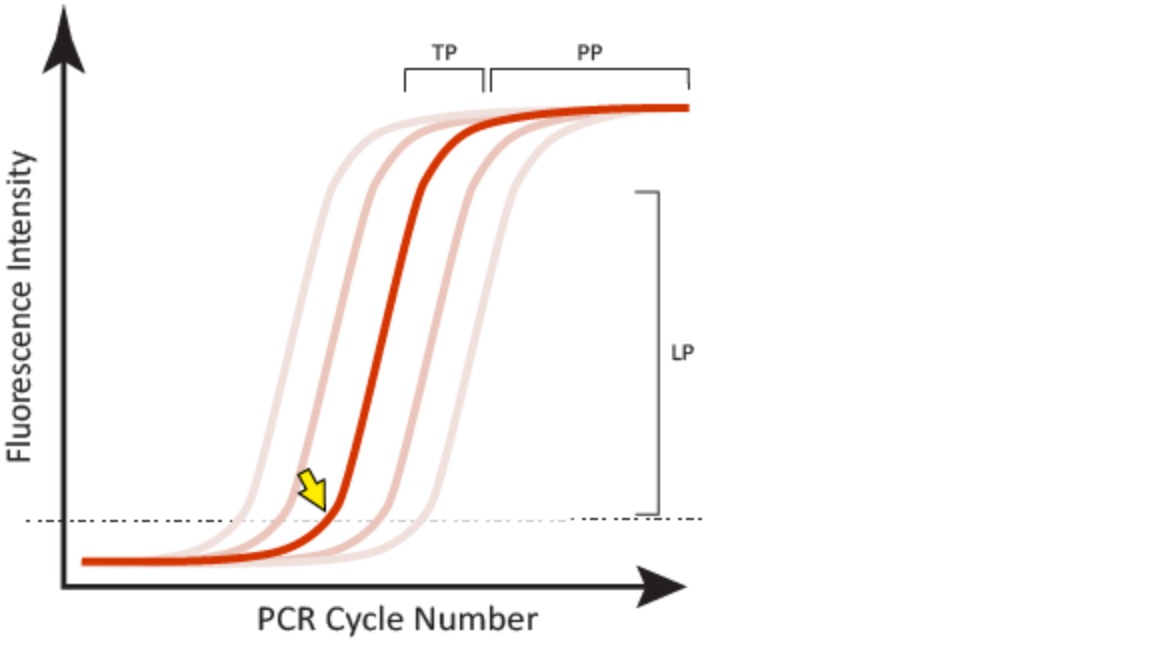

Figure 1. Examples of satisfactory sigmoidal amplification curve shape when considering an assay's fluorescent signal output. The crossing point or threshold cycle (C_T) is indicated (yellow arrow); it is the value at which fluorescence levels surpass a predefined (usually set during validation, or arbitrary) threshold level as shown in this normalized linear scale depiction. LP-log-linear phase of signal generated during the exponential part of the PCR amplification; TP-a slowing of the amplification and accompanying fluorescence signal marks the transition phase; PP-the plateau phase is reached when there is little or no increase in fluorescent signal despite continued cycling.