Mix for N number of reactions:

assay mixture A components	1 Reaction (Total volume : 18 µℓ)	Volumes for N specimens (µℓ)
Buffer 5 X	4 µℓ	4 x (N+5%N)
Primer mix 10 X	2 µℓ	2 x (N+5%N)
Probe mix 10 X	2 µℓ	2 x (N+5%N)
Enzyme mix 20 X	1 µℓ	1 x (N+5%N)
Nuclease-free water	7 µℓ	7 x (N+5%N)

) Mix by pipetting and perform short spin. Pipette 16 µℓ of each assay mixture into applicable wells. Cover and transfer the plate into sample processing area.

*Sample preparation should be performed at sample processing area

i) Add 4 µℓ of the extracted RNA, or positive control, or nuclease-free water to the wells prefilled with the assay mixtures. If more substrate volume must be included, adjust water volume accordingly.

RNA extract Volume + Water Volume should equal to 11 .

e.g., If 5 µℓ need to be included in each reaction just add 11-5= 6 µℓ of nuclease free water instead of 9 in each reaction and aliquot 15 µℓ of assay mixture in each well before adding template.

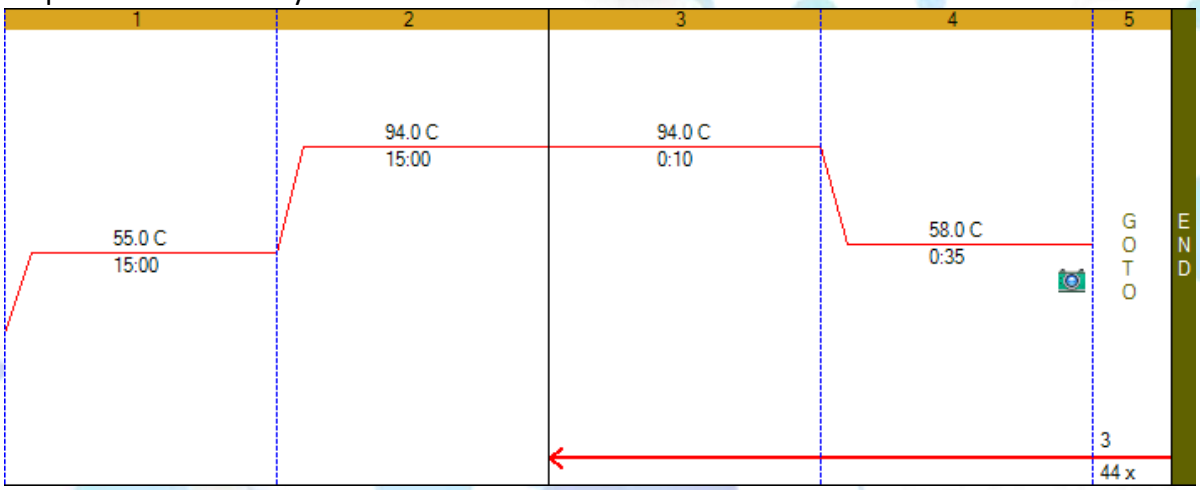
7. Reaction conditions

For each PCR instrument and software, enter the following assay settings for the COVID-19 qPCR Multi Kit.

① Enter the reaction volume 20 µL and modify PCR reaction conditions presented in the following table.

Step	Temperature	Time	Cycle
RT	55°C	15 min	1
Incubation	94°C	10-15 min *	1
Amplification	94°C	10 sec	45
	58°C **	35 sec	

* For optimal quantification keep 15 min. 10 min incubation results to reduced amplification efficiency.



Measure fluorescence at 58°C (FAM, HEX and Cy5 channels).

8. Quality control

All lots are tested with Exact diagnostics positive control (see reference) and EURM-019 (see reference).

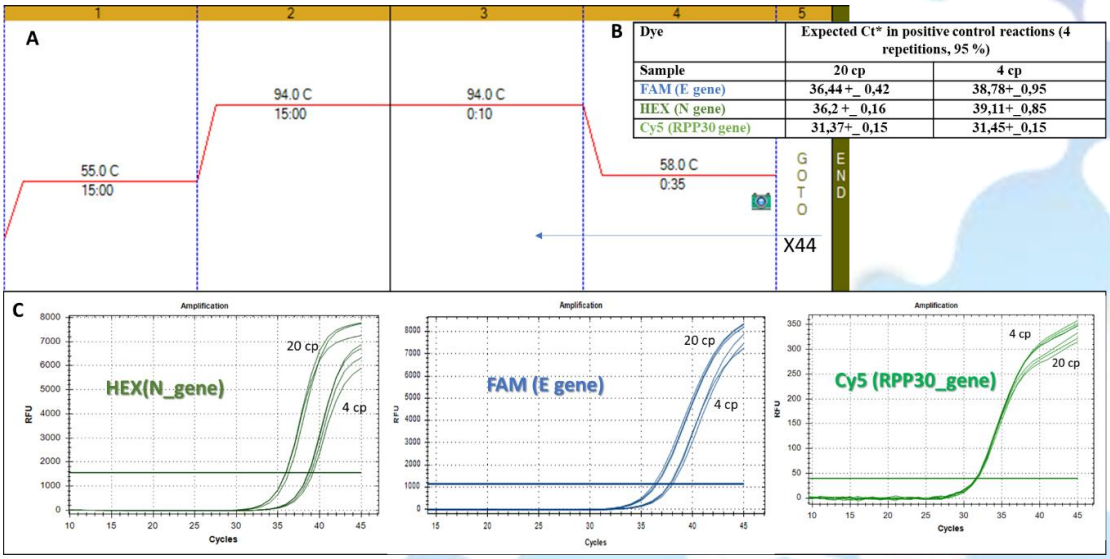
Limit of detection verification

Triplicates of 20 cps and quadruplicate of 4 cps per reaction are analyzed. In each reaction 10 ng of total RNA from HeLa cells and 80 ng of carrier RNA (see reference) are included.

QC standards of each lot are as follow:

Dye	Expected Ct* in positive control reactions (4 repetitions, 95 %)	
Sample	**20 cp**	**4 cp**
FAM (E gene)	36,44 ± 0,42	38,78+_0,95
HEX (N gene)	36,2 ± 0,16	39,11+_0,85
Cy5 (RPP30 gene)	31,37 ± 0,15	31,45+_0,15

* Automatic base line-Biorad CFX Manager software)



Dye	Expected Ct* in positive control reactions (4 repetitions, 95 %)	
Sample	20 cp	4 cp
FAM (E gene)	36,44+_ 0,42	38,78+_0,95
HEX (N gene)	36,2 +_ 0,16	39,11+_0,85
Cy5 (RPP30 gene)	31,37+_ 0,15	31,45+_0,15

Image 1. QC_ protocol. A. The thermocycling program as depicted by CFX manager software**, B.** Table showing the average Cts and standard deviation obtained after QC run (base line was automatically adjusted by CFX manager program). **C.** Amplification curves obtained for each transcript. 20 and 4 copies of exact diagnostics template were added in reaction.

Standard Curve using EURM-019 template

Serial dilutions of EURM-019 ranging from 10^5 to 10 copies (in quadruplicates) were prepared and subjected to RT-PCR analysis with our reagents. It is important to note that in contrast to plasmids, where no reverse transcription is needed, the chimeric RNA EURM-019 was used as template for standard curve analysis, taking into account both enzymatic activities.

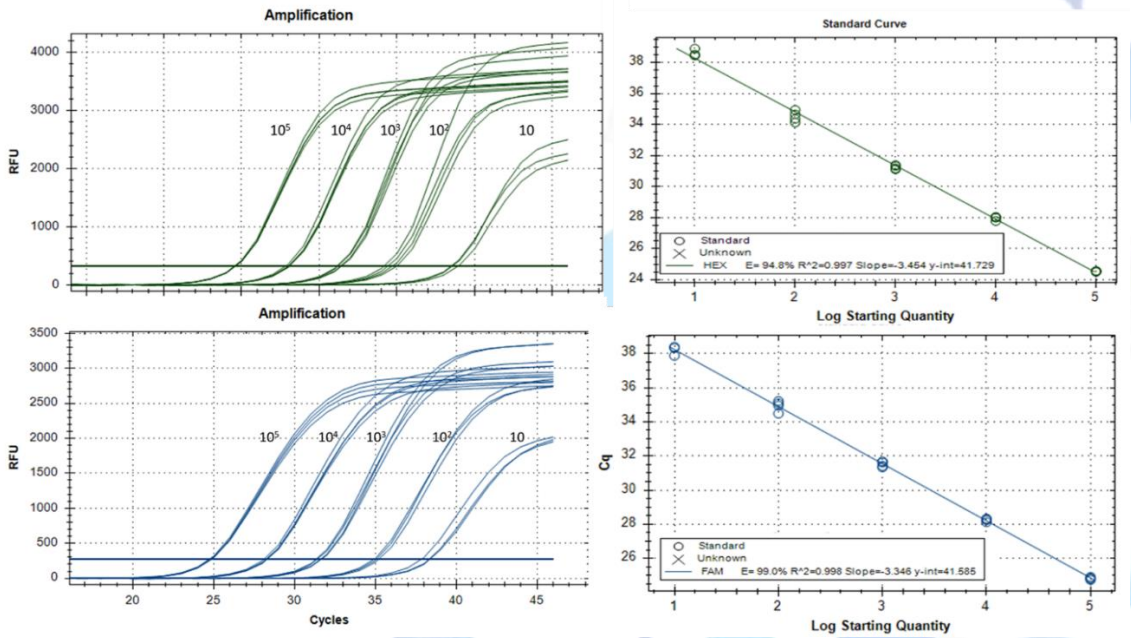


Image 2. Standard Curve analysis. HEX(N_gene) and FAM (E_gene) slopes and standard curve after RT-PCR analysis of EURM-019 serial dilutions. Efficiencies were auto calculated by CFX manager to 94.8 % and 99 % for HEX and FAM respectively.

9. Interpretation of Results

Ct Value.

A reaction is considered positive if there is a sigmoidal amplification curve with a Ct value no higher than 40 at threshold value (threshold value should be ~ in the middle of exponential phase)

Reaction	Ct value for either SARS COV 2 genes
Positive	**≤ 40**
Negative	**>40**

Control

For Positive control reaction supplied in our kit (2µl) the Ct values obtained after 4 replicates are the following:

Dye	Expected Ct* in positive control reactions (4 repetitions, 95 %)
FAM (E gene)	32,38 +_ 0,46
HEX (N gene)	32,1 +_ 0,30
Cy5 (RPP30 gene)	32,2 +_ 0,79

This product is intended for research use only. The purchaser is responsible to acquire the respective authorization certificates for its particular use.

Sample result interpretation.

Sample Analysis			
HEX (N_gene)	FAM (E_gene)	Cy5 (RPP30_gene)	Interpretation
+	+	+	Positive for SARS COV2
+/-	+	+	Positive for SARS COV2
+	+/-	+	Positive for SARS COV2
-	-	+	Negative for SARS COV2
-	-	-	Invalid result**
+	+/-	-	Potentially Positive for SARS COV2/ Very high viral titer in a poor-quality RNA sample*
+/-	+	-	Potentially Positive for SARS COV2/ Very high viral titer in a poor-quality RNA sample*

***Repetition of RNA extraction and new RT-PCR reaction is strongly suggested. If the result is persistent re-sampling and re-testing is advised.**

**** Repetition of RNA extraction and new RT-PCR reaction is strongly suggested. If the result is persistent re-sampling and re-testing is advised. Sampling error leading to total inhibition may be the causes of this result.**

Important Note: « + » : Ct value ≤ 40, « - » : Ct value >40

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