EQ SARS COV2 One Step RT qPCR kit for wastewater, 200 rxns (Cat No: RN016S)

Description:

EQ SARS COV2 One Step RT qPCR kit for wastewater contains a 5x reaction buffer optimized for sensitive and specific detection of SARS COV2 RNA species from various sources and purity backgrounds. Enzymes come separately in 20x and 200x formulations to provide flexibility and allow user to optimize depending on the purity of the substrate of interest (mainly on the presence of PCR inhibitors). The 10x SARS COV2 Oligos mix contains a combination of primers and probe for SARS COV2 N gene (modified USA CDC N2 primer/probe set), that allows the qPCR to detect SARS COV2 transcripts at HEX/VIC channel. Optimizations on wastewater extracts samples guarantee the reproducible and efficient qPCR reactions close to the expected limit of detection (<10 copies per reaction). It can reliably detect and quantify as low as 10 copies per reaction, based on a standard curve produced by analyzing EURM019 dilutions, with efficiency ~ 90-110 %.

Reagents supplied:

EQ SARS COV2 One Step RT qPCR kit for wastewater, 200 rxns (Cat No: RN016S)		
Cat No	**Description**	**Tubes No.**
BR030-1	5x One Step RT-qPCR buffer, 1 ml	1
PD015-0.225	20x Hot Start Taq DNA polymerase, 0.225ml	1
RN015-0.025	200x Reverse Transcriptase, 0.025ml	1
BR060-0.425	10x SARS COV2 Oligos mix, 0.425 ml	1
BR033- 1	Nuclease Free Water, 1 ml	2

Shelf life:
1 year

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Standard 20 µl reaction:

Component		Concentration of Stock solutions	1 reaction of 20 µl	Final Concentration/Quantity (20 µl assay)
1	**5x One Step RT-PCR buffer**	5x	4 µℓ	1x
2	**10x SARS COV2 Oligos mix**	10x	2 µℓ	1x
3	**20x Hot Start Taq DNA polymerase**	20x	1 µℓ	1x
4	**200x Reverse Transcriptase**	200x	0.1 µℓ*	1x
5	**Template RNA****	5-10^5 copies of target sequence/µl	2-4 µℓ	N/A
6	**Nuclease Free Water**	N/A	Till 20 µℓ	N/A

***0.08-0.12 µl may be added depending on the substrate used. For Reference Labs wastewater extracts analysis, 0.1 µl has been used extensively used. Do not add more than needed, because PCR inhibition effect may be caused by the excess of Reverse Transcriptase enzyme in the reaction.**
****2 µl of RNA extracts is recommended. In case of high concentration of PCR inhibitors, perform 1:10 dilution of the original wastewater extract sample and add 2-4 µL per reaction. 2 µL of wastewater extracts from Reference Labs have been extensively used in our analysis**.

Optional: Depending on the wastewater quality, the region or the season collected, treatment of wastewater extracts with PCR inhibitors removal kits like the ones provided by ZYMO Research (https://zymoresearch.eu/collections/onestep-pcr-inhibitor-removal-kits), may be important to obtain accurate, quantifiable results. In order to verify the necessity of this type of treatment, test 4 independently extracted samples, collected over 2-4 weeks, and compare the quantification of the 1:10 dilution of either treated or untreated sample. If the difference obtained is lower than 30 % then that treatment may be omitted from the analysis.

Proposed assays

There are two assays proposed for the analysis.

The initial assay

The initial standard assay is very important to be able:

A. To establish the quantification equivalence between the current kit and the standard method used so far (if applicable)
B. To check the necessity of PCR inhibitor removal kits.

To that end, the reactions that are needed to be set are the following:

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i. **Standard curves 10^5- 10^1 in triplicates** → 15 reactions

ii. **1:10 dilution of wastewater extracts in triplicates** → 3 x number of samples

iii. **(Optional)** If PCR inhibitor test needs to be performed, incubate half the amount of the extracted wastewater samples with PCR inhibitor removal kit, then dilute 1:10 and analyze in triplicates → 3 x number of samples with PCR inhibitor removal treatment (same samples included in ii).

iv. **Two negative control reactions that contain Nuclease Free Water instead of template.**

After the reactions have been completed, produce the standard curve and estimate efficiency and R^2. If the efficiency is between 90-110% and R^2 value above 0.98 then proceed to quantification. One may exclude reactions that do not conform with the expected values.
(Optional/ When applicable) Quantify samples with or without PCR inhibitor removal treatment and see whether there is a difference greater than 30 %. If there is, then the PCR inhibitor removal kit may be necessary before the RT-qPCR analysis for the accurate quantification of the viral load in the wastewater sample. If in both cases, treatment or not, a similar quantity is estimated for the same sample, then the PCR inhibitor removal step may be omitted.
If the quantification of this kit needs to be compared with a different method, then the same sample needs to be analyzed again with the different method at the same freeze-thaw cycle, meaning that previous quantity record of the sample, may deviate greatly due to RNA degradation and not the difference in kits'/methods' quantification efficiency.

The Routine/minimal assay

After the initial run, the routine assay for wastewater analysis may contain:

i. **A standard curve of 4 logs in duplicates of 10^2-10^5 copies/reaction** → 8 reactions
ii. **1:10 dilutions of wastewater extracts in triplicates** → 3 x number of wastewater samples
iii. **2 negative control reactions** → 2 reactions

The "routine/minimal assay" is more susceptible to handling errors and is only recommended if repetition between replicates is high (figure 1). For best results follow the standard curve preparation as described in the "Initial assay" section.

Optimized component addition order for master mix preparation

First add water and then continue by adding the buffer, the oligos, the 20x Hot Start Taq DNA polymerase and finally the 200x Reverse Transcriptase. While adding each enzyme to the master mix, pipette up and down for 5-10 times (to dissolve glycerol) and then add the next one. Perform one final mix with a mild vortex (1000-1200 rpm) until glycerol is completely dissolved. Aliquot the appropriate volume and add

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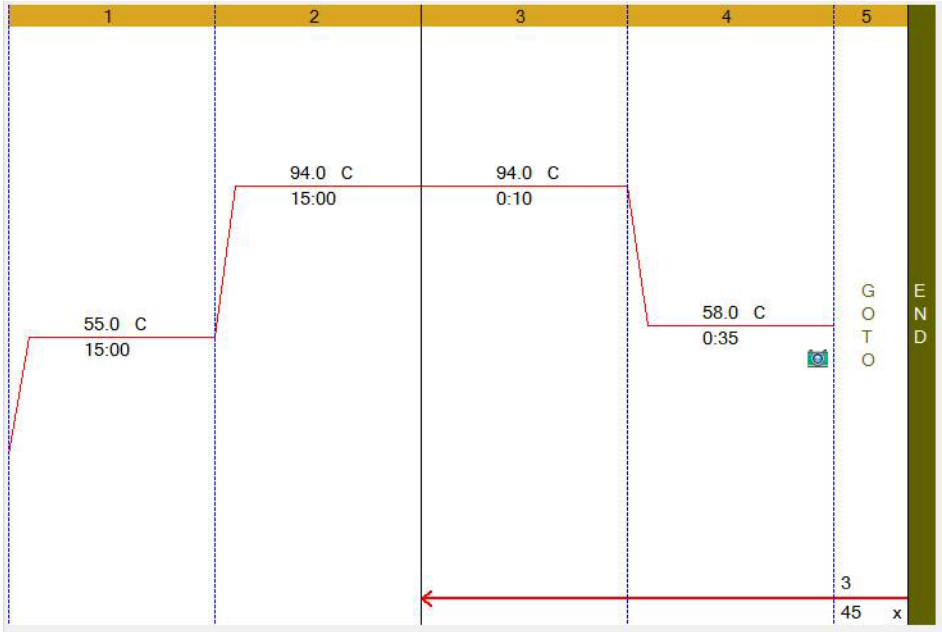
water to the negative control reactions and close the caps. Then proceed to the samples addition and close the caps and finally add template to the standard curve reactions and close the caps.

Always calculate an additional 5% of each component when calculating volumes for master mix preparation to avoid pipetting errors. E.g. if you wish to set 20 reactions, then you should multiply the volume needed for one reaction by 21 for each component. If you wish to prepare 10 reactions, then multiply by 10.5 etc.

One Step-RT recommended conditions.



1	55.0 C for 15:00
2	94.0 C for 15:00
3	94.0 C for 0:10
4	58.0 C for 0:35
	+ Plate Read
5	GOTO 3 , 45 more times
	END

Step 1: RT reaction.

Step 2: RT inactivation/ Hot Start Taq DNA polymerase activation.

Initial activation for 15 minutes at 94°C is important for reactions with low PCR template copies and for obtaining the maximum efficiency in qPCR setups. DO NOT USE FOR LESS THAN 10 min!!

Step 3: Cycle denaturation.

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Step 4: Annealing/Extension step. The recording is on HEX/VIC channel.

After the analysis finishes, set the baseline in the area where maximum efficiency is obtained. For Bio-Rad platforms, set at 150-350 Relative fluorescent units. For other platforms adjust according to your standard practice.

Additives that improve robustness

In cases of samples with low viral content near the limit of detection (~5-10 copies/µl of wastewater sample) and/or high concentration of inhibitors one may also add extra DTT or T4gp32 protein to improve reactions' efficiency. Final concentrations in qPCR reactions should be **1mM for DTT** and **0.05 µg/µl for T4gp32**. An example of the reaction setup including both additives may be found below:

	Component	Concentration of Stock solutions	1 reaction of 20 µl	Final Concentration/Quantity (20 µl assay)
1	**5x One Step RT-PCR buffer**	5x	4 µℓ	1x
2	**10x SARS COV2 Oligos mix**	10x	2 µℓ	1x
3	**DTT (Customer provided)**	100 mM	0.2 µℓ	1 mM
4	**Gp32 (Customer provided)**	10 µg/µl	0.1 µℓ	0.05 µg/µl
5	**20x Hot Start Taq DNA polymerase**	20x	1 µℓ	1x
6	**200x Reverse Transcriptase**	200x	0.1 µℓ	1x
7	**Template RNA***	5-10^5 copies of target sequence/µl	2-4 µℓ	N/A
8	**Nuclease Free Water**	N/A	Till 20 µℓ	N/A

***2 µl of RNA extracts is recommended. In case of high concentration of PCR inhibitors, perform 1:10 dilution of the original wastewater extract sample and add 2-4 µL per reaction. 2 µL of wastewater extracts from Reference Labs have been extensively used in our analysis.**

Recommended suppliers:

- For **DTT** any product that is compatible with molecular biology applications (example A2948/Applichem, DTT Molecular biology grade or equivalent)

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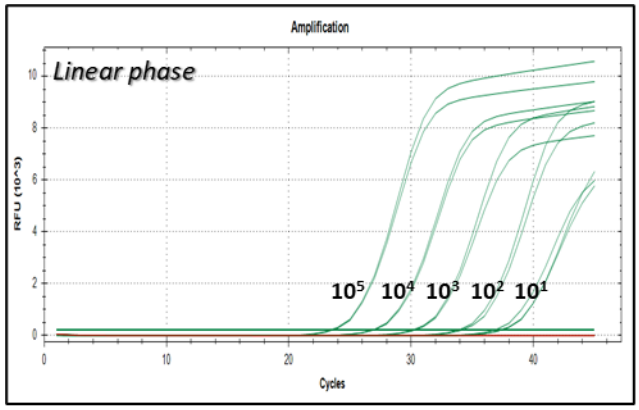
- For **Gp32** use any provider that is compatible with molecular biology applications. The inhouse assays have been performed with cat number **M300S or L from New England Biolabs** (T4 Gene DTT may reverse mildly the inhibition potential of the sample whereas both should be used when high inhibition is suspected.

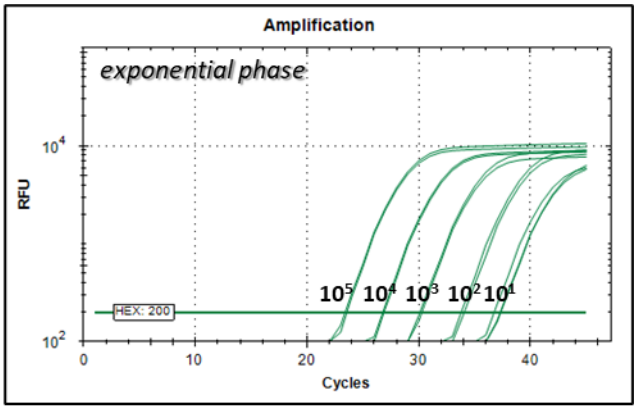
- Other Quality Controls:
  Certified as DNase-RNase free using fluorescently labeled RNase and DNase probes. Efficiency between 90-110%, with R2 value >0.99 of each lot is estimated by a standard curve assay produced over 5 logs of EURM019 substrate (https://crm.jrc.ec.europa.eu/p/EURM-019)

- Storage conditions
  Store at -20oC ± 5oC

SARS COV 2 standard EURM019

A. Initial standard curve assay





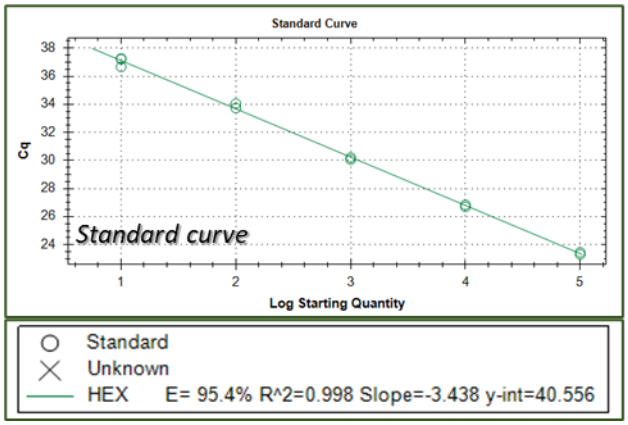


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Figure 1. SARS COV2 standard curves produced by EQ SARS COV2 One Step RT qPCR kit for wastewater (Cat No: RN016S). The standard curves were produced by analyzing the EURM019 SARS COV 2 standard over 5 logs (example of initial assay), having as a given that the EURM019 stock solution contains 10^8 copies of SARS COV2 chimeric transcripts/µl (detailed info for EURM019 used may be found at https://crm.jrc.ec.europa.eu/p/EURM-019). Since the quantification of the EURM019 standard, performed by digital droplet PCR by manufacturer, produced results between 6 - 7.6 x 10^7 copies/µl depending on the primer set used, the actual number of copies that were introduced in each reaction may be 24-40 % less than indicated in the figure. The results were automatically produced by CFX Maestro software. The reactions were incubated to either CFX96 or OPUS96 qPCR machines with very similar results.

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