Title: STUDY OF BACTERICIDAL AND VIRUCIDAL ACTIVITY AGAINST SARS-CoV-2 of a book sterilizer based on Ultraviolet C.

1. Description of the equipment

The equipment on which the test was carried out is of Korean origin and is used for the disinfection of books and documents: Ultraviolet C Book Sterilizer SELF CLEAN. It consists of a cabinet in which are the germicidal Ultraviolet C lamps and 12 supports on which to place the books and notebooks. In addition, it has a wind generation system from the bottom to the back where a HEPA filter is located in which any particle larger than 0.20 µm is retained, viruses are retained by this filter.

Its use is very simple: the passenger compartment door is opened and the notebooks are placed on the supports, the door is closed, the sterilization time is regulated and it is started.

Figure 1. Image of the Ultraviolet C Ray Book sterilizer Self Clean book sterilizer at AENER facilities.

2. Objective

Check the effectiveness of the sterilizer of SELF CLEAN books, bactericidal and virucidal, based on a combination of air and Ultraviolet C radiation in three sterilization periods, 30 seconds, 1 minute and 2 minutes.

3. Materials and methodology

3.1 Bactericidal Test Materials

- *Pseudomonas aeruginosa* CECT110T strain: *Pseudomonas aeruginosa* (Schroeter 1872) Migula 1900 Strain designation: NCIMB 8295. Genetic Data: 16S rRNA sequence: HE978271. Whole genome sequence: GCA_001042925.1

Culturing conditions:

Primary culture medium: Nutrient Agar/Agar II: beef extract: 1 g; Yeast extract: 2 g, Peptone: 5 g; NaCl: 5g; Add 15 g in 1 litre of distilled water, pH 7.2.

Cultivation temperature: 36°C

Incubation Time: 24 h

Atmospheric needs: aerobic

Figure 2: *Pseudomonas aeruginosa* CECT 110T in CFC Agar medium at 24 h, courtesy of the Spanish Type Culture Collection.

- Cultivator with agitation and temperature control
- Sterile Erlenmeyers
- Biosan mini-bioreactor
- Falcon tubes with membrane for Mini-reactor
- Sterile 1.5 ml cryotubes
- 1-40 µL pipette and sterile filter tips
- Rodac contact plates for surface sampling with CN medium, selective for *Pseudomonas aeruginosa*.
- Cetrimide-Nalidixic Agar Medium: Pancreatic Gelatin Peptone: 16.0 g; Casein hydrolysate: 10.0 g; Cetrimide: 0.2 g; Mg Cl 1.4 g; K2SO4 10.0 g; in 1 litre of distilled water Agar 15.0 PH 7.1. After autoclaving and cooling add 0.015 g/L of Nalidixic Acid.
- Autoclave
- Celin 1003 spectrophotometer.

- Optical microscope
- Ultrasonic bath
- Neu-Bauer chamber for cell counting

3.2 Materials for virucidal testing against SARS-CoV-2

- Positive control: ALL-WHO-CDC-Genes n CoV-Control-Plasmid PEX-A128-nCOV- all 1 µg Lyophilisate.

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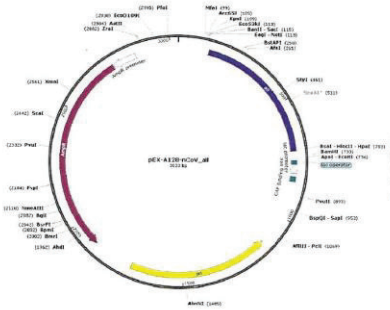
Corona COVID-19 positive control plasmid

Target Name:	**All-WHO-CDC-Genes**	**Internal Name:**	**nCoV_all**
Plasmid Name:	pEX-A128-nCoV_all	**Gene Size:**	583 bp
Vector Backbone:	pEX-A128	**Antibiotic Selection:**	Ampicillin
Cloning:	via Type IIS restriction enzymes	**Quantity:**	1µg lyophilized plasmid

Plasmid Map



5' Restriction Site: n/a
3' Restriction Site: n/a
Cloning: via Type IIS restriction enzymes
(Type IIS sites not present in final plasmid)

MCS of pEX-A128-nCoV_all

```
GGAGCAGACAAGCCCGTCAGGGCGCGTCAGCGGGTGTTGGCGGGTGTCG
GGGCTGGCTTAACTATGCGGCATCAGAGCAGATTGTACTGAGAGAAAGG
caattgGGTACCgagctcGCGGCCGCAAGC>All WHOCDCGenes>ACC
TGCTTTTGCTCGCTTGGATCCgaattcAAAGGTGAAATTGTTATCCGCT
CACAATTCCACACAACATACGAGCCGGAAGCATAAAGTGTAAAGCCTG
```

Please Note:

Verify sequence after each cloning step.

The plasmid DNA has been lyophilised. We recommend to dissolve it in water.

Eurofins Genomics

Christian Barth
Technician QC, Gene Synthesis

Controlled and Released

00-042QR_A128 V1.1 / 20170502

Figure 3. Plasmid certificate with all recommended sequences by the World Health Organization (WHO) used as a positive control.

- Sampling kit with swab and tube containing 3 ml of RNA preservative and virus inactivator solution.
- RNA Extraction Kit: Viral RNA GPSpin Kit (Genetic PCR Solutions)
- SARS-CoV-2 determination and quantification kit: CoVID-19 dtec-RT-qPCR TestGenetic Detection of Severe acute respiratory syndrome coronavirus 2. (Genetic PCR Solutions). Kit Validated by the Carlos III Health Institute (100% sensitivity and specificity)
- StepOne Real-Time PCR System: Applied Biosystems
- StepOne Real-Time PCR System Software: Applied Biosystems
- 100 µL and 20 µL sterile pipettes and sterile tips with filters
- Cabinet with Ultraviolet C
- Microeppedort 100 µL tubes for PCR
- 1 mL eppendorft tubes
- Mini centrifuge
- Vortex

3.3 Methodology

3.3.1 Preparation of the *Pseudomonas aeruginosa* 110T strain

The *Pseudomonas aeruginosa* 110T strain is thawed for 30" at 36°C and grown in Nutrient Agar for 24 hours to check its viability and specificity Sunday, July 5).

A colony is taken and cultivated in nutrient broth at 37°C 24 hours in a cultivator with agitation at 120 rpm (Monday, July 6).

From here, 2 ml are taken and passed to a Falcon with 10 ml of Nutrient Broth and placed in the Biosan Mini Bioreactor to control its growth curve and perform the cell count. It is cultivated at 36°C for 24 h (Tuesday, July 7) to obtain 10^8 CFU/ml (Colony Forming Units per mL). At the same time, we are taking data on the optical density and correlating it with the optical density to read the results.

3.3.2. Cell quantification

To quantify the number of bacteria, a regression line was carried out between the optical density (abscissa axis) and the number of bacteria CFU/mL on a logarithmic scale (ordinate axis), for this, serial dilutions were made starting from the *Ps. aeruginosa* solution with 10^8 CFU/mL up to 10^1 and its absorbance at a wavelength of 660 nm was measured in the spectrophotometer.

This allows us to obtain an equation that fits a line and will facilitate the calculation of the CFU/mL that we obtain in the various controls and treatments.

The following figure shows the table with the obtained Absorbances (Optical Density, O.D.) and the CFU/mL and the graph for their representation with the regression equation obtained and the

regression coefficient R^2. For its representation, the CFU/mL data have been logarithmically transformed.

O.D. (nm)	CFU / mL
0,642	1,0E+08
0,584	1,0E+07
0,499	1,0E+06
0,435	1,0E+05
0,358	1,0E+04
0,284	1,0E+03
0,187	1,0E+02
0,087	1,0E+01

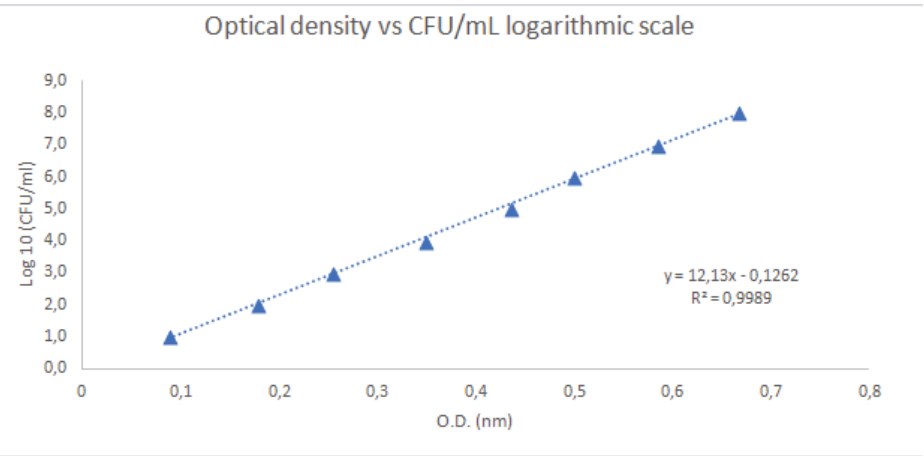


Figure 4. Equation for calculating cells of *Ps. Aeruginosa*.

3.3.3. Preparation and quantification of the plasmid

The plasmid with the SARS-CoV-2 sequences is lyophilized and for its use it must be resuspended with 1 ml of ultrapure water in the same vial.

For its quantification, the commercial GPS Kit was used with which the calibration line was carried out with the standards that come in the same kit, starting from an initial concentration of 10^6 copies/mL and whose regression equation is seen in the following figure.

The resuspended plasmid gave us a CT of 10 that corresponds to 10^6 copies/mL.

The equation obtained will allow us to quantify the number of copies of the sequence of the SARS-CoV-2 virus inserted in the control plasmid used for the test and after the three treatments.

Copies/mL	Log 10	CT
1000000	6	**10,2500**
100000	5	**15,0727**
10000	4	**19,0227**
1000	3	**23,4500**
100	2	**27,9963**
10	1	**32,2975**

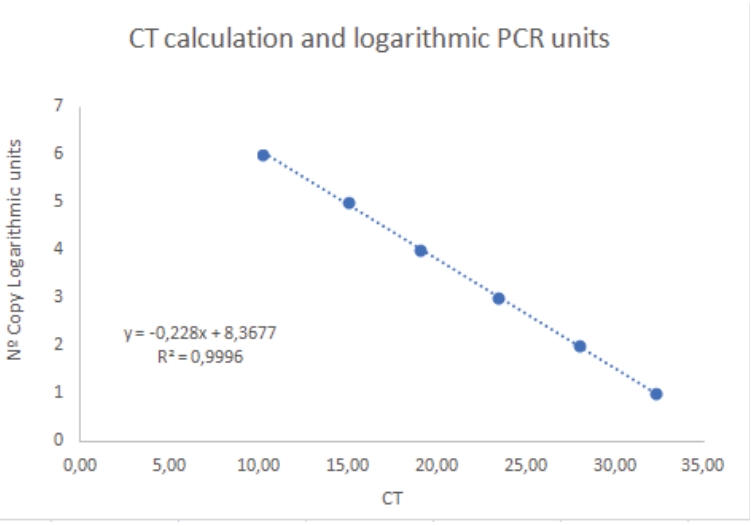


Figure 5. Correlation between the CT obtained in RT-qPCR and the number of copies in logarithmic units.

3.3.4. Experimental design for taking samples after treatments

Four new notebooks have been used for the test:

Control notebook: to perform positive and negative controls.

Rest of notebooks for one of the treatments: 30", 1' and 2'.

Figure 6. Notebooks on which the tests have been carried out. The pink notebook corresponds to the positive and negative controls carried out in the laboratory.

Two sheets of paper were taken, one in the first half of the notebook that was dedicated to the bacteria and another in the second half of the notebook that was dedicated to the virus. In each of them, 5 circles of 25 cm^2 were made and numbered to maintain a fixed position in each repetition. Positions 1, 2, 3, 4, and 5 are the labels for positive controls and for all three treatments.

Figure 7. Arrangement of the points to apply inoculums and take samples.

3.3.4.1 Negative controls

For the negative control of *Pseudomonas aeruginosa*, a sheet was taken on which a surface plate with CN medium was applied and cultivated at 36°C for 24 h.

A sample was also taken from the surface of the paper with a swab and inserted into the tube with virus inactivating liquid and RNA preservative for negative virus control.

3.3.4.2. Positive controls

For the positive control of the bacteria, on a sheet with 5 circles, 20 µL of bacterial inoculum with 10^8 CFU/mL ($2.0*10^6$ CFU) was applied to each surface of the circle and left soak through the paper for a few seconds. After this, samples were taken with the contact plates. The following image shows the fluorescence emitted by the bacteria under Ultraviolet light.

A second repetition of inoculation was made and the sample was taken again, but in this case, what we did was perform a count of bacteria attached to the plate to control the possible loss that occurs when the inoculum is soaked in the paper. After collecting the bacteria, the contact surface was placed in a 90 mm diameter Petri dish containing 10 ml of nutrient broth, as well as the initial inoculum with which we carried out the calibration line, and they were placed in the ultrasound bath for 5 minutes. 2 ml of the broth with the bacteria in suspension of the positive controls were transferred to a glass cuvette of 1 cm light path and its optical density was measured at 660 nm.

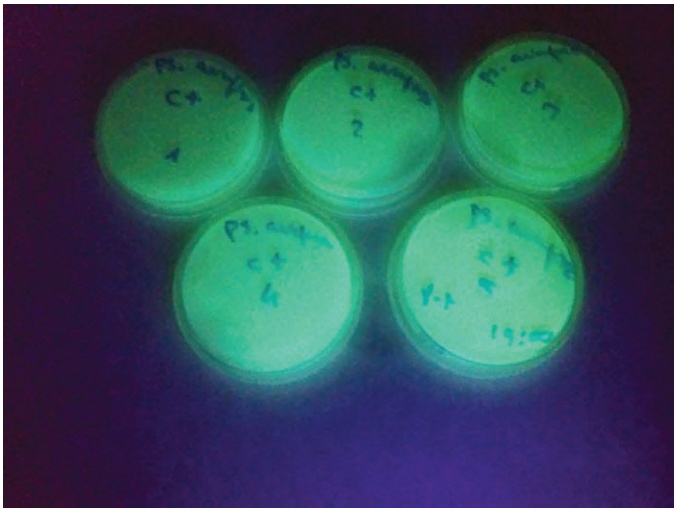


Figure 8. Positive controls for *Pseudomonas aeruginosa* under Ultraviolet light. This bacterium emits fluorescence under Ultraviolet. The entire surface is covered in bacteria.

As with the bacteria, 20 µL of each inoculum was placed in each of the circles, it was spread with the tip of the pipette and the sample was subsequently taken with the swab and placed in the tube with the virus inactivator. and the RNA preservative until the PCR test.

3.3.4.3 Sampling after treatments

In the AENER company the following day the same tests were performed as with the positive controls, in the other three notebooks, treatment 1 (30"), treatment 2 (1') and treatment 3 (2'). Inoculums were made on a sheet of *Pseudomonas aeruginosa* and on another sheet in the second half of the notebook, the viral plasmid was left to soak and the notebook was subsequently placed in a book sterilizer 30", 1' and 2', respectively.

After each treatment, samples of the bacteria were taken, on the one hand, by means of a rolling contact plate with CN medium and, on the other, by swabbing the virus samples.

Contact plates were transported cold at 4°C to the laboratory and tubes with the plasmid swab in a refrigerator without refrigeration.

To check the bacteria removal performance, the plate was placed with the surface facing down in another Petri one with 20 mL of nutrient broth in an ultrasound bath for 5 minutes and vortexed for 30" to extract the inoculum from each of the treatments. The number of bacterial cells was obtained by measuring the absorbance 660 nm and the equation obtained above.

For the virus, the number of copies detected after each treatment was taken as a decrease compared to the positive controls without treatment, measured by RT-qPCR.

3.3.5. Calculations

Equation to obtain the performance of decreased bacterial and viral load.

Bacteria/virus decline rate (%)

$$R\ (\%) = [(A - B) / A] * 100$$

R: rate of decline

A: CFU/mL of the positive control or copies number of the positive control

B: CFU/mL of the treatment or copies number of the treatment

4. Results

4.1 Results with P*seudomonas aeruginosa*

Table 1: Count of CFU/mL bacteria in each of the positions in which the inoculum was placed.

	Positive Control	Treatment 30"	Treatment 1'	Treatment 2"
Position 1	1,7E+06	4,6E+04	2,2E+04	5,7E+02
Position 2	1,3E+06	1,5E+04	3,1E+03	1,7E+02
Position 3	1,8E+06	8,0E+04	2,3E+04	1,3E+03
Position 4	1,1E+06	1,6E+04	2,1E+03	1,2E+02
Position 5	8,1E+05	3,4E+04	4,0E+03	3,0E+02
Average	**1,3E+06**	**3,8E+04**	**1,1E+04**	**4,8E+02**

Table 2: Rate of decrease in % of the bacterial load in each of the positions in which the inoculum was placed.

	Treatment 30"	Treatment 1'	Treatment 2"
Position 1	97,269	98,714	99,966
Position 2	98,882	99,759	99,987
Position 3	95,486	98,714	99,929
Position 4	98,480	99,802	99,989
Position 5	95,849	99,502	99,963
Average	**97,132**	**99,192**	**99,964**

4.2 Results with plasmid with SARS-CoV-2 sequences.

The CT of the negative control was 38.91, less than 10 copies per mL.

Table 3: Number of plasmid copies with SARS-CoV-2 in each position and treatment.

	Positive Control	Treatment 30"	Treatment 1'	Treatment 2"
Position 1	2,3E+04	5,7E+03	1,0E+03	1,3E+02
Position 2	3,9E+04	4,5E+03	1,4E+03	8,9E+01
Position 3	5,6E+04	3,2E+03	2,1E+03	3,0E+02
Position 4	9,7E+04	2,2E+03	1,0E+03	1,2E+02
Position 5	7,8E+04	6,0E+03	5,8E+02	1,8E+02
Average	**5,9E+04**	**4,3E+03**	**1,2E+03**	**1,6E+02**

Table 4: Rate of decrease in % of the viral load in each of the positions in which the inoculum was placed.

	Tratamiento 30"	Tratamiento 1'	Tratamiento 2"
Position 1	75,383	95,601	99,429
Position 2	88,681	96,358	99,773
Position 3	94,189	96,162	99,467
Position 4	97,693	98,967	99,873
Position 5	92,285	99,258	99,775
Average	**92,617**	**97,895**	**99,721**

5. Discussion and Conclusions

The 2019 coronavirus disease pandemic (COVID-19) has caused a major disaster worldwide, devastating global health and economic metrics. On January 30, 2020, the World Health Organization (WHO) declared SARS-CoV-2 as a global public health emergency [1]. COVID-19 is characterized by high morbidity and low mortality, therefore it represents a great threat, particularly for immunocompromised people, the elderly and individuals with pre-existing health problems. The data so far suggests that the virus has a case fatality rate of around 1% [2]. Therefore, it is several times more severe than typical seasonal influenza, with a severity somewhere between the 1957 (0.6%) and 1918 (2%) influenza pandemics [2], but it appears to be lower than SARS (9.5%) and average Eastern respiratory syndrome (34.4%) [3]. Additionally, the average person infected with COVID-19 spreads the disease efficiently to two or three others: an exponential rate of increase. There is also strong evidence that the virus can be transmitted by infected individuals who are asymptomatic or who experience mild symptoms [4].

The methods to sterilize in Microbiology are very varied from autoclaving at 121°C 20' filtration through pore sizes less than 0.20 mm or the use of germicidal Ultraviolet C radiation. Sterilization is especially important when we talk about pathogenic microorganisms for humans, that is, those that reproduce in our body and cause us diseases, as has happened in recent months with SARS-CoV-2. The use of Ultraviolet C in Microbiology laboratories is very common to work in sterile environments for which the eradication time ranges from 30 to 45 minutes, a period of time in which all other bacteria, viral or genetic material disappears.

This pandemic has conditioned our lives in such a way that solutions are sought in all areas so that economic activity does not have to stop, but always guaranteeing the best possible working and customer service conditions.

The equipment in which the tests have been carried out pursues this aim, that books and documents can be sterilized in a relatively short time so that they can continue to be used by different users and thus reduce the risk of transmission of the virus through the contact with the paper.

As a result of the bactericidal test, we observed that by increasing the exposure time, the bacterial load is reduced, reducing up to 4 logarithmic units on average, resulting in a decrease in bacterial load of 99.96%. If we look at the position of the samples, it can be seen that positions 2 and 4 tend to have a lower amount of CFU/mL than 1, 3 and 5 because the first ones are towards the right outer margin of the sheet, while that the other three are towards the centre of the notebook.

As for the viral load, the same pattern is observed as for *Pseudomonas aeruginosa*, except that in this case the decrease is two logarithmic units of the positive control to the treatment lasting two minutes, which represents an average reduction of 99.72%.

However, we must bear in mind that we are always using high bacterial and viral loads and that the reduction of 3 or 4 logarithmic units, by itself, gives us significant reductions when we speak in

percentage terms. However, it is important to know if that charge that still remains on the paper means a transmissible dose and this will depend on the microorganism or virus in question.

In the case of *Pseudomonas aeruginosa* that is a ubiquitous pathogen, the minimum infective dose is currently unknown [5], therefore, we cannot affirm that the remaining doses that remain on paper are infectious or not.

And in the case of SARS-CoV-2 there is also no evidence on the minimum infective dose, it seems that with several hundred copies it is already infective, however, for its transmissibility it seems that more copies above 10^5 are needed [6]. Thus, at least the 2 minute treatment would decrease the transmissibility of the virus.

As a conclusion of the trial, the system used reduces the risk of spreading both the bacteria tested and the virus by more than 99%, especially when the duration of the treatment is longer.

On the other hand, the equipment with which it has been worked has the advantage of combining two disinfection systems, on the one hand the germicidal Ultraviolet C radiation and, on the other hand, an air current that drags particles towards a HEPA filter that prevents the spread of viruses and bacteria out of the machine compartment. This means that this machine needs maintenance to achieve more optimal results, such as changing filters when necessary and UV-C lamps depending on the hours of use to maintain the effectiveness of the disinfection system.

2020 July 13, Monday
Technical Director and R+D+i

Ph.D. Mª Angélica García Álvaro

ANNEXES

Unidad de Virus Respiratorios y Gripe
Laboratorio de Referencia e Investigación en Virus Respiratorios
Centro Nacional de Microbiología

INFORME DE VALIDACIÓN DEL SISTEMA COMERCIAL CoVID-19 dtec-RT-qPCR Test F100 format DE LA EMPRESA GENETIC PCR SOLUTIONS PARA LA DETECCIÓN DEL NUEVO CORONAVIRUS SARS-CoV2 EN MUESTRAS RESPIRATORIAS

Responsable del informe: Jesús Oteo Iglesias. Director del Centro Nacional de Microbiología. Instituto de Salud Carlos III.

Fecha del informe: 10 de marzo de 2020

Objetivo.

Validación de un sistema comercial de la empresa GENETIC PCR SOLUTIONS (GENETIC ANALYSIS STRATEGIES S.L.) para la detección del coronavirus SARS-CoV2 en muestras respiratorias. El sistema es el COVID-19 dtec-RT-qPCR Test, en su formato de 100 reacciones, que consiste en una PCR en tiempo real para la amplificación de un solo gen de SARS-CoV2, no incluye análisis de un gen alternativo.

Metodología.

El sistema COVID-19 dtec-RT-qPCR Test se ha probado con un panel de 80 muestras respiratorias (exudados nasofaríngeos) anonimizadas del biobanco del Centro Nacional de Microbiología (CNM). Este panel incluye 41 muestras positivas y 39 muestras negativas previamente caracterizadas según metodología recomendada por la OMS y optimizada en el CNM, mediante dos PCRs en tiempo real según procedimientos de referencia en cuanto a su extracción y amplificación (1).

Resultados.

Los resultados obtenidos mediante el sistema COVID-19 dtec-RT-qPCR Test (Tabla 1) muestran un total de 41 muestras positivas y 39 negativas, con un 100% de sensibilidad y un 100% de especificidad.

En la Tabla 1 se detallan los resultados obtenidos para el sistema analizado en cada una de las muestras probadas y en comparación con los resultados de referencia.

Conclusiones

En este estudio, el sistema probado ha mostrado una sensibilidad y especificidad del 100% en la detección de material genético de SARS-CoV2 en muestras respiratorias.

Referencias

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Optical density values obtained and the calculation in CFU/mL

	C +	CFU/mL Log 10	CFU/mL
P1	0,524	6,2	1,7E+06
P2	0,515	6,1	1,3E+06
P3	0,526	6,2	1,8E+06
P4	0,508	6,0	1,1E+06
P5	0,498	5,9	8,1E+05

	t= 30"	CFU/mL Log 10	CFU/mL
P1	0,395	4,7	4,6E+04
P2	0,354	4,2	1,5E+04
P3	0,415	4,9	8,0E+04
P4	0,358	4,2	1,6E+04
P5	0,384	4,5	3,4E+04

	t=1'	CFU/mL Log 10	CFU/mL
P1	0,368	4,3	2,2E+04
P2	0,299	3,5	3,1E+03
P3	0,370	4,4	2,3E+04
P4	0,285	3,3	2,1E+03
P5	0,308	3,6	4,0E+03

	t=2'	CFU/mL Log 10	CFU/mL
P1	0,238	2,8	5,7E+02
P2	0,194	2,2	1,7E+02
P3	0,266	3,1	1,3E+03
P4	0,183	2,1	1,2E+02
P5	0,215	2,5	3,0E+02

Ct values obtained from RT-qPCR

Negative control: CT = 39.91 <10 copies/mL

	Controls +	Ulog	copies/mL
P1	17,56	4,36402	2,3E+04
P2	16,54	4,59658	3,9E+04
P3	15,88	4,74706	5,6E+04
P4	14,83	4,98646	9,7E+04
P5	15,24	4,89298	7,8E+04

	t= 30"	Ulog	copies/mL
P1	20,23	3,75526	5,7E+03
P2	20,69	3,65038	4,5E+03
P3	21,3	3,5113	3,2E+03
P4	22,01	3,34942	2,2E+03
P5	20,12	3,78034	6,0E+03

	t= 1'	Ulog	copies/mL
P1	23,51	3,00742	1,0E+03
P2	22,85	3,1579	1,4E+03
P3	22,09	3,33118	2,1E+03
P4	23,54	3,00058	1,0E+03
P5	24,58	2,76346	5,8E+02

	t= 2'	Ulog	copies/mL
P1	27,4	2,1205	1,3E+02
P2	28,14	1,95178	8,9E+01
P3	25,85	2,4739	3,0E+02
P4	27,54	2,08858	1,2E+02
P5	26,85	2,2459	1,8E+02

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