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Test with the certificate of GLPs
(Good Laboratory Practices)
No. 2/19-C.VAL. General Directorate of Pharmacy and Medical Devices of the Health Department of the Valencian Region. Spain

Virucidal test with the product "ZOTAL ZERO" against Coronavirus (Based on EN 14476: 2014 + A2: 2019 Guideline) with deviations from the guideline

Report
Registration No.: D/20/232

1. **Laboratory identification** ……. Instituto Valenciano de Microbiología.

2. **Client identification …**……..…… LABORATORIOS ZOTAL, S.L.
   Address ……..…..…… Carretera Nacional 630, Km. 809
   41900- Camas.

3. **Sample identification** (information provided by the customer)

- Product name……. ZOTAL ZERO.
- Batch number……. P002L01F20/03-1.
- Expiration date……... March 2025.
- Manufacturer (supplier)……. LABORATORIOS ZOTAL
- Date of manufacturer……. Not indicated.
- Storing conditions ……. Room temperature.
- Diluent of the product recommended by the manufacturer……... Water.
- Active(s) Substance(s) and its concentration (s)…… Biphenol-2-ol 4%, Chlorocresol 0.9%-Glycolic acid 0.1%.
- Conditions of use……... Surfaces.
- Concentrations ordered for the assay…. 5%, 2.5% and 1%.

IVAMI is not responsible for customer-supplied information.

4. Information about sample reception.

- Date of reception of order with test conditions ...……..…….. 2020/03/23.
- Date of reception of the product……. 2020/03/23.
- Aspect of the received product……... Brown transparent fluid in a metallic commercial container.

5. Testing method

Procedure **DESIN-6225** (Based on EN 14476: 2014 + A2: 2019 guideline).

6. Experimental conditions

- Assay period…….. 2020/03/24 to 2020/04/08.
- Assay temperature……. 37ºC ± 1ºC.
- Titration method …….. $TCID_{50}$ (Tissue Culture Infective Dose 50%).
- Product concentrations for the assay…. 5%, 2.5% and 1%.
- Contact time……... 30 minutes.
- Contact temperature……... 20ºC ± 1ºC.
- Procedure to stop product cytotoxicity.. Molecular sieving.
- Procedure to stop product activity Cooling with ice.
- Solvent of the product used in the assay…….. Sterile hard water.
- Aspect of the dilutions of the product… Transparent.
- Stability of the mixture (interfering substance and product diluted in sterile hard water)……..……... Stable.
- Interfering substance:
    - Internal control of dirty conditions in the presence of bovine serum albumin 3 g/L.
    - Dirty conditions in the presence of bovine serum albumin 3 g/L plus 3 mL erythrocytes 3 mL/L.
- Identification of the origin of viral strains and number of passes…….. Coronavirus 229E (ATCC VR-740) aliquot: 2019/03/04 passage 2.
- Cell lines (name, origin, number of passes)……..... MRC-5 ref. FTMR, working aliquot 3, passages 16, 18 and 19.

7. Validation of assay results

Coronavirus 229E (ATCC VR-740)

Titre of the viral suspension for the virus control (30 minutes):

- Dirty conditions.....log $10^{-5.82}$
- Internal control of dirty conditions..... log $10^{-5.82}$

Cytotoxicity level (5%).....log $10^{-0.5}$

Maximum level of virus inactivation detectable (difference between the titre of the viral suspension and the cytotoxicity level):

- Dirty conditions……...log $10^{-5.32}$
- Internal control of dirty conditions……..……log $10^{-5.32}$

Reference test (formaldehyde 1.4%)

Cytotoxicity level of formaldehyde 0.7%……..... log $10^{-0.5}$

Viral quantification in the reference test (formaldehyde) after 15 minutes and with Coronavirus 229E.....log$10^{-2.74}$

Confidence interval

Title of virus with 95% confidence interval with Coronavirus 229E (30 minutes)

- Dirty conditions ……log $10^{-5.82 \pm 0.34}$
- Internal control of dirty conditions ……...log $10^{-5.82\pm 0.34}$

Reduction with the confidence interval of 95 % ……See table 1.

Sensitivity of cells to virus

- Viral quantification of Coronavirus 229E with cells not treated with “ZOTAL ZERO” disinfectant …….....log$10^{-5.91}$
- Viral quantification of Coronavirus 229E with cells treated with the “ZOTAL ZERO” disinfectant…….……...……..….....log$10^{-5.66}$

Note: only can be used to determine the infectivity of cells, those dilutions which: a) show a low degree of cellular destruction (< 25% of cell monolayer) and b) produce a reduction of the title of the virus <1$\log_{10}$.

Control of the effectivity of the disinfectant detection activity

- Viral quantification of Coronavirus 229E after 30 minutes on bath ice without exposing the virus to the "ZOTAL ZERO" disinfectant ……..……..…….. $log10^{-5.99}$
- Viral quantification of Coronavirus 229E exposing the virus to "ZOTAL ZERO" disinfectant and incubated 30 minutes on ice bath…….. $log10^{-5.66}$

Note: The difference between decimal logarithm of titre without exposing the virus to the product and of the test suspension should be ≤0.5

8. Special remarks

- All controls and validation were between the basic limits.
- No concentration showed a log reduction lower than 4 log.
- One concentration at least showed a log reduction higher than ≥4 log.

9. Assay results

9.1 Description

The disinfectant product**, "ZOTAL ZERO",** batch **P002L01F20/03-1,** under dirty conditions, diluted at 5%, 2.5% and 1% and during 30 minutes of exposure, **shows** virucidal activity against Coronavirus 229E (ATCC VR-740), with a reduction ≥ 5.32 ± 0.34 $TCID_{50}$ for the three concentrations, when the activity is assayed according with the internal procedure DESIN-6255 based on the EN 14476: 2014 + A2: 2019 guideline, with deviations.

9.2 Tables of results and graphics

See tables 1 and 2 and figure 1.

10. Conclusion

The disinfectant product "**ZOTAL ZERO**", batch **P002L01F20/03-1**, under dirty conditions (bovine serum albumin 3 g/L plus 3 mL erythrocytes 3 mL/L), diluted at 5%, 2.5% and 1%, requested by the customer, and during 30 minutes of exposure, shows virucidal activity against Coronavirus 229E (ATCC VR-740), when the activity is assayed according with the internal procedure DESIN-6255 based on the EN 14476: 2014 + A2: 2019 guideline, with deviations from the guideline since a non-active concentration has not been tested.

Tests performed only with Coronavirus strain 229E, does not allow to conclude that the product tested shows a general virucidal activity, but only that it shows activity against Coronaviruses.

Note 1: The results obtained correspond to the product received in this laboratory.
Note 2: The information that depend on the information received from the client and are not facilitated by the same one, shown as "not provided".

Bétera (Valencia), April 14, 2020.

Signed. Miguel Ángel Fernández
Responsible Technician
(Investigator)

Quality Assurance Review:

The assay development and the results obtained have been supervised by the Director of the study.

The Quality Assurance Director has inspected the development of the assay, proving that has been realized following the proper procedure and using the adequate media, materials and reagents, following as well the Good Laboratory Practices (GLPs) and the final report contains the primary data obtained.

Signed. Ruth Novella
Responsible for the Laboratory Area
(Study Director)

Signed. Encarnación Esteban
Technical Director
(Quality Assurance Director)

Reference:

- EN 14476: 2014 + A2: 2019 Guideline. Virucidal quantitative suspension test for chemical disinfectants and antiseptics used in human medicine. Test method and requirements (Phase 2/Step 1).

Table 1. Results of activity of the product "**ZOTAL ZERO",** batch **P002L01F20/03-1** with Coronavirus 229E (ATCC VR-740) under dirty conditions.

Product	Concen-tration*	Interfering substance	Cytoto-xicity level	log_{10} $TCID_{50}$ after…..				Reduction with the confidence interval of 95 % after 30 minutes
				0 min	5 min	15 min	30 min	
ZOTAL ZERO	5%	3 g/L BSA + 3 mL/L erythrocytes	0.5	-	-	-	0.5	≥5.32 ± 0.34
	2.5%		0.5	-	-	-	0.5	≥5.32 ± 0.34
	1%		0.5	-	-	-	0.5	≥5.32 ± 0.34
ZOTAL ZERO	5%	3 g/L BSA	0.5	-	-	-	0.5	≥5.32 ± 0.34
	2.5%		0.5	-	-	-	0.5	≥5.32 ± 0.34
	1%		0.5	-	-	-	0.5	≥5.32 ± 0.34
Formaldehyde	0.7% (w:v)	NA	0.5	NR	3.82	2.74	NR	NA
Virus control	NA	3 g/L BSA + 3 mL/L erythrocytes	NA	6.08	-	-	5.82	NA
Virus control	NA	3 g/L BSA	NA	5.99	-	-	5.82	NA
Virus control Formaldehyde	0.7% (w:v)	NA	NA	5.91	NR	5.83	NR	NA

Control of sensitivity of cells to virus (difference between decimal logarithm of titre using treated and untreated cells) …….$log10^{-0.25}$
Control of the effectiveness of the disinfectant detection activity (difference between decimal logarithm of titre without exposing the virus to the product and of the test suspension)……..$log10^{-0.33}$

NA: not applicable; NR: not realized
Times recommended by Guideline for surfaces: maximum 5 or 60 minutes
Times recommended by Guideline for instruments: maximum 60 minutes
Times recommended by Guideline for Hygienic treatment of hands by friction and hygienic handwashing: between 30 or 120 seconds
PBS: phosphate buffered saline; BSA: bovine serum albumin.
Virucidal activity exists when the titre of virus shows a reduction ≥4 log.
*: see Special remarks to understand the values of these concentrations.

Table 2. Results of the activity of the product **"ZOTAL ZERO"**, batch **P002L01F20/03-1**, with Coronavirus 229E (ATCC VR-740) (Assay of titration with 12 wells), under dirty conditions.

Product	Concen-tration *	Interfering substance	Time of contact (min)	Dilutions (log10)[a,b]							
				1	2	3	4	5	6	7	8
ZOTAL ZERO	5%	3 g/L BSA + 3 mL/L erythrocytes	30	0000 0000 0000	0000 0000 0000	0000 0000 0000	0000 0000 0000	0000 0000 0000	0000 0000 0000	0000 0000 0000	NR
	2.5%		30	0000 0000 0000	0000 0000 0000	0000 0000 0000	0000 0000 0000	0000 0000 0000	0000 0000 0000	0000 0000 0000	NR
	1%		30	0000 0000 0000	0000 0000 0000	0000 0000 0000	0000 0000 0000	0000 0000 0000	0000 0000 0000	0000 0000 0000	NR
ZOTAL ZERO	5%	3 g/L BSA	30	0000 0000 0000	0000 0000 0000	0000 0000 0000	0000 0000 0000	0000 0000 0000	0000 0000 0000	0000 0000 0000	NR
	2.5%		30	0000 0000 0000	0000 0000 0000	0000 0000 0000	0000 0000 0000	0000 0000 0000	0000 0000 0000	0000 0000 0000	NR
	1%		30	0000 0000 0000	0000 0000 0000	0000 0000 0000	0000 0000 0000	0000 0000 0000	0000 0000 0000	0000 0000 0000	NR
Cytotoxicity	5%	3 g/L BSA + 3 mL/L erythrocytes	NA	0000 0000 0000	0000 0000 0000	0000 0000 0000	0000 0000 0000	0000 0000 0000	0000 0000 0000	0000 0000 0000	NR
Virus control	NA	3 g/L BSA + 3 mL/L erythrocytes	0	4444 4444 4444	4444 4444 4444	4444 4444 4444	4444 4444 4444	4444 4444 4444	0320 2221 0030	0000 0000 0000	NR
			30	4444 4444 4444	4444 4444 4444	4444 4444 4444	4444 4444 4444	3022 2333 3222	0000 2011 2000	0000 0000 0000	NR
Virus control	NA	3 g/L BSA	0	4444 4444 4444	4444 4444 4444	4444 4444 4444	4444 4444 4444	2332 2330 3222	0221 0110 0120	0000 0000 0000	NR
			30	4444 4444 4444	4444 4444 4444	4444 4444 4444	4444 4444 4444	3202 2233 3232	0000 0102 2100	0000 0000 0000	NR
Formaldehyde	0.7 (w/v)	NA	5	4444 4444 4444	4444 4444 4444	2330 0233 3222	0000 0020 1110	0000 0000 0000	0000 0000 0000	0000 0000 0000	NR
			15	4444 4444 4444	2020 0232 2322	0000 0010 2110	0000 0000 0000	0000 0000 0000	0000 0000 0000	0000 0000 0000	NR
Control of formaldehyde cytotoxicity	0.7 (w/v)	3 g/L BSA + 3 mL/L erythrocytes	NA	0000 0000 0000	0000 0000 0000	0000 0000 0000	0000 0000 0000	0000 0000 0000	0000 0000 0000	0000 0000 0000	NR
Virus control formaldehyde	0.7 (w/v)	NA	0	4444 4444 4444	4444 4444 4444	4444 4444 4444	4444 4444 4444	2332 0232 3322	0100 0021 1012	0000 0000 0000	NR
			15	4444 4444 4444	4444 4444 4444	4444 4444 4444	4444 4444 4444	2322 0302 3222	0212 0002 0110	0000 0000 0000	NR

Sensitivity control of cells to virus	NA	NA	Cells not treated	CCCC CCCC CCCC	CCCC CCCC CCCC	CCCC CCCC CCCC	CCCC CCCC CCCC	CCC0 CCCC CCCC	0C00 0CCC 0CC0	0000 0000 0000	NR
			Cells treated	CCCC CCCC CCCC	CCCC CCCC CCCC	CCCC CCCC CCCC	CCCC CCCC CCC0	C0C0 CCCC CCCC	0000 0C0C C0C0	0000 0000 0000	NR
Effectiveness control of the disinfectant detection activity	NA	3 g/L BSA + 3 mL/L erythrocytes	Without ZOTAL ZERO	CCCC CCCC CCCC	CCCC CCCC CCCC	CCCC CCCC CCCC	CCCC CCCC CCCC	CCCC CCC0 CCCC	0CCC CC00 0CC0	0000 0000 0000	NR
			With ZOTAL ZERO	CCCC CCCC CCCC	CCCC CCCC CCCC	CCCC CCCC CCCC	CCCC CCCC CCCC	CC0C CCCC 0CCC	0000 CCC0 00C0	0000 0000 0000	NR

a): 1 to 4, virus present and grade of cytopathic effect in 12 units of cellular culture, or grade of cellular lesions in the cytotoxicity assay.
C = cytopathic effect with presence of virus (in this case and according to guideline does not take into account the degree of cytopathic effect only, the presence or absence of the same).
0 = no virus present or absence of cellular lesions in the cytotoxicity assay; NA: not applicable; NR: not realized; BSA: Bovine serum albumin; PBS: phosphate buffered saline.
sec: seconds; min: minutes.
*: see Special remarks to understand the values of these concentrations.

Figure 1. Results of the activity of the product **"ZOTAL ZERO"**, batch **P002L01F20/03-1**, at 5%, 2.5% and 1% concentration under dirty conditions with Coronavirus 229E (ATCC VR-740).

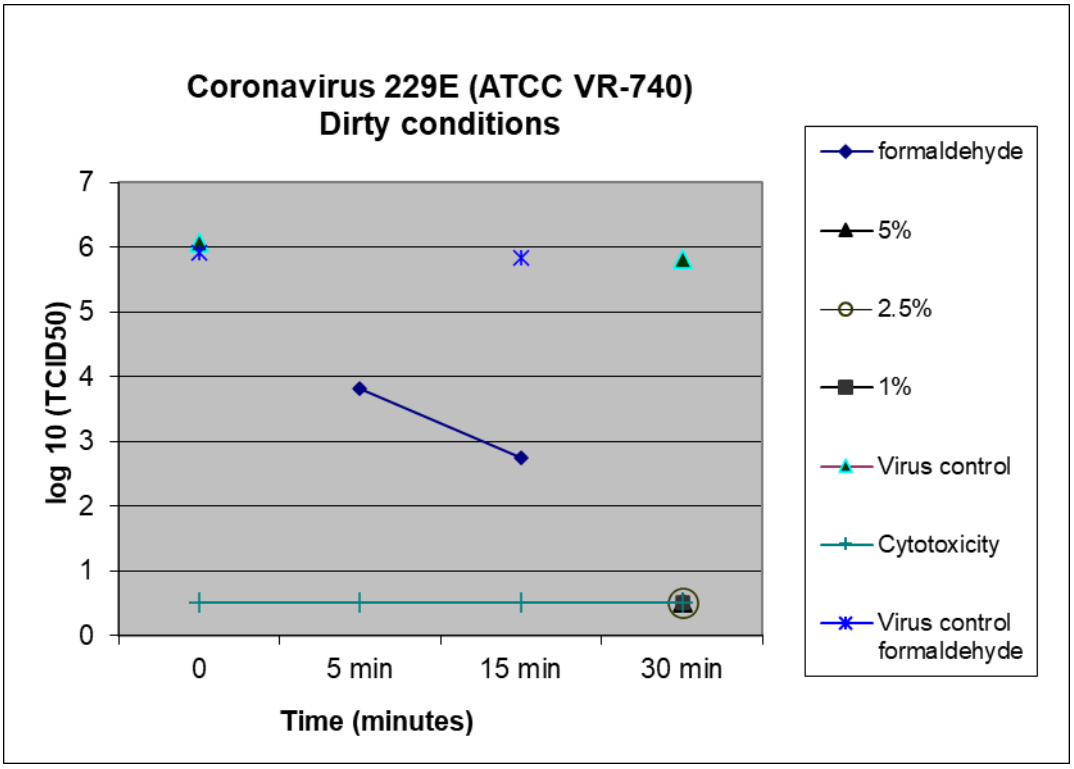

Figure 1.1 Results of the activity of the product **"ZOTAL ZERO"**, batch **P002L01F20/03-1**, at 5%, 2.5% and 1% concentration under internal control of dirty conditions with Coronavirus 229E (ATCC VR-740).