Test Report No.: VX-TR-24-0031
Copy No.: 1

DETERMINATION OF THE BACTERICIDAL ACTIVITY (EN 1276) OF HAND CLEANING WIPES NO

Lab No.:	VX-246-24-0001
Sample Name:	**Hand Cleaning Wipes NO**
Method:	EN 1276:2019 (E) Chemical disinfectants and antiseptics – Quantitative suspension test for the evaluation of bactericidal activity of chemical disinfectants and antiseptics used in food, industrial, domestic and institutional areas – Test method and requirements (phase 2, step 1)
Client:	Dr.Raymund Schuster Consulting Sambugaweg 12 DE 69190 Walldorf Germany
Sample Receipt Date:	22 December 2023
Report Date:	23 February 2024

Kuala Lumpur, 23 February 2024

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Head of Microbiological Testing

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Materials and Method

Quantitative suspension test for the evaluation of bactericidal activity of chemical disinfectants and antiseptics used in the food, industrial, domestic, and institutional areas according to EN 1276:2019 (E)

1. Testing laboratory identification

Viroxy Sdn. Bhd.
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2. Sample identification

2.1	Sample name:	Hand Cleaning Wipes NO
2.2	Batch no.:	14122023_1
2.3	Product appearance:	Clear, colourless solution
2.4	Manufacturer:	ECS Cleaning Solutions GmbH Wolfener Strasse 32-34 DE 12681 Berlin Germany
2.5	Active substances per 100 g:	17.83 g Ethanol 1.92 g Propan-2-ol
2.6	Sample receipt date:	22 December 2023
2.7	Storage conditions:	Room temperature
2.8	Product diluent:	Not applicable; ready-to-use product

3. Experimental conditions

3.1	Testing period:	21 February 2024
3.2	Test organism(s):	*Enterococcus hirae* ATCC 10541 *Escherichia coli* ATCC 10536 *Pseudomonas aeruginosa* ATCC 15442 *Staphylococcus aureus* ATCC 6538
3.3	Concentration / contact time:	100.00 %* / 2 minutes
3.4	Loading:	0.30 g/L bovine albumin solution
3.5	Test temperature:	20 °C ± 1 °C
3.6	Counting method:	Pour plate Spread plate (for *P. aeruginosa* only)
3.7	Incubation period:	24 hours, 36 °C ± 1 °C

4. Test method and its validation

4.1	Testing method:	Dilution-neutralization
4.2	Inactivation combination:	30.00 g/L Tween 80 30.00 g/L Saponin 3.00 g/L Lecithin 1.00 g/L L-Histidine 1.00 g/L L-Cysteine in tryptone soya broth

The results of validation tests A, B, and C proved the viability of the method in all cases.

5. Test results

The results are stated in Tables A and B.

6. Conclusion

Hand Cleaning Wipes NO showed the required microbial reduction of ≥5.0 $\log_{10}$ against test strain(s) *Enterococcus hirae* ATCC 10541, *Escherichia coli* ATCC 10536, *Pseudomonas aeruginosa* ATCC 15442, and *Staphylococcus aureus* ATCC 6538 in accordance with EN 1276:2019 (E) at 100.00 %* concentration after 2 minutes under the stated conditions. According to the simple acceptance decision rule†, there is a minimal risk of false acceptance.

Kuala Lumpur, 23 February 2024

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7. Note

Bactericidal activity – the capability of a product to produce a reduction in the number of viable bacteria belonging to reference strains under defined conditions by at least 5 orders (10^5).

$R = N_0/N_a$ = the reduction in viability, or $\lg R = \lg N_0 - \lg N_a$

* The product can only be tested at 80.00 % concentration or less, as some dilution always occurs when test organisms and interfering substance are added.

† The decision rule applied is simple acceptance rule with no guard band and up to 50 % risk of false acceptance or rejection. This rule has been determined by the laboratory and agreed with the client prior to testing.

Table A: Evaluation of the bactericidal activity of Hand Cleaning Wipes NO on test strain(s) according to EN 1276

Product: Hand Cleaning Wipes NO
Loading: 0.30 g/L bovine albumin solution

Test strain: *Enterococcus hirae* ATCC 10541

N	V_{C1}	V_{C2}	Test suspension, N
10^{-6}	>330	>330	N: 3.10×10^8 N_0: 3.10×10^7 lg N_0: **7.49**
10^{-7}	33	29	

Test concentration (%) / contact time (min)	Dilution	V_{C1}	V_{C2}	Test procedure, N_a $N_a = \bar{x} \times 10$
100.00* / 2	10^0	<14	<14	N_a: $<1.40 \times 10^2$ lg N_a: **<2.15** lg R: **>5.35**

Test strain: *Escherichia coli* ATCC 10536

N	V_{C1}	V_{C2}	Test suspension, N
10^{-6}	>330	>330	N: 4.15×10^8 N_0: 4.15×10^7 lg N_0: **7.62**
10^{-7}	43	40	

Test concentration (%) / contact time (min)	Dilution	V_{C1}	V_{C2}	Test procedure, N_a $N_a = \bar{x} \times 10$
100.00* / 2	10^0	<14	<14	N_a: $<1.40 \times 10^2$ lg N_a: **<2.15** lg R: **>5.47**

Test strain: *Pseudomonas aeruginosa* ATCC 15442

N	V_{C1}	V_{C2}	Test suspension, N
10^{-6}	>660	>660	N: 3.90×10^8 N_0: 3.90×10^7 lg N_0: **7.59**
10^{-7}	38	40	

Test concentration (%) / contact time (min)	Dilution	V_{C1}	V_{C2}	Test procedure, N_a $N_a = \bar{x} \times 10$
100.00* / 2	10^0	<14	<14	N_a: $<1.40 \times 10^2$ lg N_a: **<2.15** lg R: **>5.44**

Test strain: *Staphylococcus aureus* ATCC 6538

N	V_{C1}	V_{C2}	Test suspension, N
10^{-6}	>330	>330	N: 4.70×10^{8} N_0: 4.70×10^{7} lg N_0: **7.67**
10^{-7}	48	46	

Test concentration (%) / contact time (min)	Dilution	V_{C1}	V_{C2}	Test procedure, N_a $N_a = \bar{x} \times 10$
100.00* / 2	10^{0}	<14	<14	N_a: $<1.40 \times 10^{2}$ lg N_a: **<2.15** lg R: **>5.53**

* The product can only be tested at 80.00 % concentration or less, as some dilution always occurs when test organisms and interfering substance are added.

Table B: Control tests and method validation for Table A

Test strain	Validation suspension	Validation of experimental conditions	Neutralizer toxicity control	Method validation control
E. hirae ATCC 10541	N_{V0}: 9.20×10^1	A: 1.01×10^2	B: 9.10×10^1	C: 1.06×10^2
E. coli ATCC 10536	N_{V0}: 1.14×10^2	A: 1.38×10^2	B: 1.12×10^2	C: 1.37×10^2
P. aeruginosa ATCC 15442	N_{V0}: 1.50×10^2	A: 1.53×10^2	B: 1.78×10^2	C: 1.75×10^2
S. aureus ATCC 6538	N_{V0}: 1.54×10^2	A: 2.25×10^2	B: 2.01×10^2	C: 1.93×10^2

Note

cfu: Colony forming units

V_C: Number of cfu counted per 1.0 ml sample

$\bar{x}$: Average V_{C1} and V_{C2} values

N: Number of cfu per ml in the test suspension

N_0: Number of cfu per ml at the beginning of the contact time

N_{V0}: Number of cfu per ml in the mixtures A and C at the beginning of the contact time

N_{VB}: Number of cfu per ml in the mixture B at the beginning of the contact time

N_a: Number of survivors per ml in the test mixture at the end of the contact time and before neutralization

A: Number of cfu per ml in the experimental conditions control

B: Number of cfu per ml in the neutralizer toxicity control

C: Number of cfu per ml in the dilution-neutralization method validation

Table C: Summary of the log reductions of the quantitative suspension test according to EN 1276

Test strain	Test concentration (%) / contact time (min)	Log reduction	Percentage reduction (%)	Associated risk†
E. hirae ATCC 10541	100.00* / 2	**>5.35 ± 0.05**	**>99.999**	Minimal risk of false acceptance
E. coli ATCC 10536	100.00* / 2	**>5.47 ± 0.05**	**>99.999**	Minimal risk of false acceptance
P. aeruginosa ATCC 15442	100.00* / 2	**>5.44 ± 0.09**	**>99.999**	Minimal risk of false acceptance
S. aureus ATCC 6538	100.00* / 2	**>5.53 ± 0.05**	**>99.999**	Minimal risk of false acceptance

* The product can only be tested at 80.00 % concentration or less, as some dilution always occurs when test organisms and interfering substance are added.

† The decision rule applied is simple acceptance rule with no guard band and up to 50 % risk of false acceptance or rejection. This rule has been determined by the laboratory and agreed with the client prior to testing.

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Efficacy of Hand Cleaning Wipes NO against *Enterococcus hirae* ATCC 10541, *Escherichia coli* ATCC 10536, *Pseudomonas aeruginosa* ATCC 15442, and *Staphylococcus aureus* ATCC 6538 in a quantitative suspension test at 20 ˚C according to EN 1276:2019 (E) under clean condition

EXPERT OPINION*

This expert opinion is based on the test report VX-TR-24-0031 dated 23 February 2024.

The bactericidal activity of the disinfectant Hand Cleaning Wipes NO of Dr.Raymund Schuster Consulting against *Enterococcus hirae* ATCC 10541, *Escherichia coli* ATCC 10536, *Pseudomonas aeruginosa* ATCC 15442, and *Staphylococcus aureus* ATCC 6538 was investigated by a quantitative suspension test according to EN 1276:2019 (E) under clean condition (0.30 g/L bovine albumin solution).

According to this suspension test, a disinfectant or a disinfectant solution at a particular concentration is considered as having bactericidal activity if the number of viable bacteria is reduced by ≥5 $\log_{10}$ (inactivation ≥99.999 %) within the recommended exposure period.

Hand Cleaning Wipes NO was examined at 20 ˚C at the concentration of 100.00 %** for the exposure time of 2 minutes. After the exposure time, the bacterial reduction exceeded 5 $\log_{10}$-steps in all assays. According to the simple acceptance decision rule†, there is a minimal risk of false acceptance. Therefore, a bactericidal activity against *Enterococcus hirae* ATCC 10541, *Escherichia coli* ATCC 10536, *Pseudomonas aeruginosa* ATCC 15442, and *Staphylococcus aureus* ATCC 6538 was measured as follows:

Clean condition 100.00 %** 2 minutes

Kuala Lumpur, 23 February 2024

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* Opinions and interpretations expressed here are outside the scope of SAMM (Laboratory Accreditation Scheme of Malaysia) accreditation.

** The product can only be tested at 80.00 % concentration or less, as some dilution always occurs when test organisms and interfering substance are added.

† The decision rule applied is simple acceptance rule with no guard band and up to 50 % risk of false acceptance or rejection. This rule has been determined by the laboratory and agreed with the client prior to testing.

Appendix 1

QAU CERTIFICATE*

The results stated in test report VX-TR-24-0031 dated 23 February 2024 were compared to the raw data of the tests and checked for correct transfer. No deviations were detected.

Kuala Lumpur, 23 February 2024

Digitally signed by AFIQ NAZRAN BIN MOHD NEZAM
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Afiq Nezam
Microbiologist

* Opinions and interpretations expressed here are outside the scope of SAMM (Laboratory Accreditation Scheme of Malaysia) accreditation.

Appendix 2 Raw data

Test Method	EN 1276:2019				
Product	Hand Cleaning Wipes NO		**Batch No.**	14122023_1	
Product Diluent	Not applicable; ready-to-use product		**Lab No.**	VX-246-24-0001	
Test Organism	*Enterococcus hirae ATCC 10541*				
Interfering Substance	0.30 g/L bovine albumin solution				
Test Temperature (°C)	20	**Incubation Temperature (°C)**		36	
Neutralizer or Rinsing Liquid	30.00 g/L Tween 80, 30.00 g/L Saponin, 3.00 g/L Lecithin, 1.00 g/L Histidine, 1.00 g/L Cysteine in Tryptone Soya Broth				
Inactivation Method	Dilution-neutralization		**Plating Method**	Pour plate	
Test Date	21/02/2024	**Analyzed By**	AWA	**Verified By**	ANE

Test and Validation Suspension

	N	V_{C1}	V_{C2}		
Test Suspension (N)	10^{-6}	>330	>330	$\bar{x}_{wm}$ = N = 3.10E+08	N_0 = N/10
	10^{-7}	33	29	lg N_0 = 7.49	7.17 ≤ lg N_0 ≤ 7.70
				Pass? **Yes**	

	V_{C1}	V_{C2}		
Validation Suspension (N_V)	95	89	N_{V0} = 92.0	N_{V0} = N_V/10
			30 ≤ N_{V0} ≤ 160	Pass? **Yes**
Validation Suspension (N_{VB})	V_{C1}	V_{C2}	N_{V0} =	N_{V0} = N_{VB}/1000
	-	-	30 ≤ N_{V0} ≤ 160	Pass? **N/A**

Validation and Control Procedures

	V_{C1}	V_{C2}		
Experimental Conditions Control (A)	103	99	A = 101.0	Pass? **Yes**
			A ≥ 0.5 x Nv/10	
Neutralizer Toxicity or Filtration Control (B)	V_{C1}	V_{C2}	B = 91.0	Pass? **Yes**
	93	89	B ≥ 0.5 x N_{VB}/1000 or Nv/10	
Method Validation (C)	V_{C1}	V_{C2}	C = 106.0	Pass? **Yes**
Concentration: 100 %	107	105	C ≥ 0.5 x Nv/10	

Test Procedure

Product Concentration	Contact Time (minutes)	Dilution	V_{C1}	V_{C2}	Na = $\bar{x}$ or $\bar{x}_{wm}$ x 10	lg Na	lg R = lg N_0 - lg Na
100 %	2	10^0	<14	<14	<1.40E+02	<2.15	>5.35
		10^{-1}	-	-			
		10^{-2}	-	-			
		10^{-3}	-	-			
		10^0					
		10^{-1}					
		10^{-2}					
		10^{-3}					
		10^0					
		10^{-1}					
		10^{-2}					
		10^{-3}					

Raw Data of Colony Count

	N^{-6}	N^{-7}	N_V	N_{VB}	A	B	C
V_{C1}	>330	33	95	-	103	93	107
V_{C2}	>330	29	89	-	99	89	105

Product Concentration	Contact Time (minutes)	Na^0		Na^{-1}		Na^{-2}		Na^{-3}	
		V_{C1}	V_{C2}	V_{C1}	V_{C2}	V_{C1}	V_{C2}	V_{C1}	V_{C2}
100 %	2	0	0	-	-	-	-	-	-

Appendix 2 Raw data

Test Method	EN 1276:2019				
Product	Hand Cleaning Wipes NO		**Batch No.**	14122023_1	
Product Diluent	Not applicable; ready-to-use product		**Lab No.**	VX-246-24-0001	
Test Organism	*Escherichia coli ATCC 10536*				
Interfering Substance	0.30 g/L bovine albumin solution				
Test Temperature (°C)	20	**Incubation Temperature (°C)**		36	
Neutralizer or Rinsing Liquid	30.00 g/L Tween 80, 30.00 g/L Saponin, 3.00 g/L Lecithin, 1.00 g/L Histidine, 1.00 g/L Cysteine in Tryptone Soya Broth				
Inactivation Method	Dilution-neutralization		**Plating Method**	Pour plate	
Test Date	21/02/2024	**Analyzed By**	AWA	**Verified By**	ANE

Test and Validation Suspension

	N	V_{C1}	V_{C2}		
Test Suspension (N)	10^{-6}	>330	>330	$\bar{x}_{wm}$ = N = 4.15E+08	N_0 = N/10
	10^{-7}	43	40	lg N_0 = 7.62	7.17 ≤ lg N_0 ≤ 7.70
				Pass? **Yes**	

	V_{C1}	V_{C2}		
Validation Suspension (N_V)			N_{V0} = 114.0	N_{V0} = N_V/10
	116	112	30 ≤ N_{V0} ≤ 160	Pass? **Yes**
Validation Suspension (N_{VB})	V_{C1}	V_{C2}	N_{V0} =	N_{V0} = N_{VB}/1000
	-	-	30 ≤ N_{V0} ≤ 160	Pass? **N/A**

Validation and Control Procedures

	V_{C1}	V_{C2}		
Experimental Conditions Control (A)			A = 138.0	Pass? **Yes**
	140	136	A ≥ 0.5 x Nv/10	
Neutralizer Toxicity or Filtration Control (B)	V_{C1}	V_{C2}	B = 112.0	Pass? **Yes**
	115	109	B ≥ 0.5 x N_{VB}/1000 or Nv/10	
Method Validation (C)	V_{C1}	V_{C2}	C = 137.0	Pass? **Yes**
Concentration: 100 %	140	134	C ≥ 0.5 x Nv/10	

Test Procedure

Product Concentration	Contact Time (minutes)	Dilution	V_{C1}	V_{C2}	Na = $\bar{x}$ or $\bar{x}_{wm}$ x 10	lg Na	lg R = lg N_0 - lg Na
100 %	2	10^0	<14	<14	<1.40E+02	<2.15	>5.47
		10^{-1}	-	-			
		10^{-2}	-	-			
		10^{-3}	-	-			
		10^0					
		10^{-1}					
		10^{-2}					
		10^{-3}					
		10^0					
		10^{-1}					
		10^{-2}					
		10^{-3}					

Raw Data of Colony Count

	N^{-6}	N^{-7}	N_V	N_{VB}	A	B	C
V_{C1}	>330	43	116	-	140	115	140
V_{C2}	>330	40	112	-	136	109	134

Product Concentration	Contact Time (minutes)	Na^0		Na^{-1}		Na^{-2}		Na^{-3}	
		V_{C1}	V_{C2}	V_{C1}	V_{C2}	V_{C1}	V_{C2}	V_{C1}	V_{C2}
100 %	2	0	0	-	-	-	-	-	-

Appendix 2 Raw data

Test Method	EN 1276:2019				
Product	Hand Cleaning Wipes NO		**Batch No.**	14122023_1	
Product Diluent	Not applicable; ready-to-use product		**Lab No.**	VX-246-24-0001	
Test Organism	*Pseudomonas aeruginosa ATCC 15442*				
Interfering Substance	0.30 g/L bovine albumin solution				
Test Temperature (°C)	20	**Incubation Temperature (°C)**		36	
Neutralizer or Rinsing Liquid	30.00 g/L Tween 80, 30.00 g/L Saponin, 3.00 g/L Lecithin, 1.00 g/L Histidine, 1.00 g/L Cysteine in Tryptone Soya Broth				
Inactivation Method	Dilution-neutralization		**Plating Method**	Spread plate	
Test Date	21/02/2024	**Analyzed By**	AWA	**Verified By**	ANE

Test and Validation Suspension

	N	V_{C1}	V_{C2}		
Test Suspension (N)				$\bar{x}_{wm}$ = N = 3.90E+08	N_0 = N/10
	10^{-6}	>660	>660	lg N_0 = 7.59	7.17 ≤ lg N_0 ≤ 7.70
	10^{-7}	38	40	Pass?	**Yes**

Validation Suspension (N_V)	V_{C1}	V_{C2}	N_{V0} = 150.0	N_{V0} = N_V/10
	148	152	30 ≤ N_{V0} ≤ 160	Pass? **Yes**
Validation Suspension (N_{VB})	V_{C1}	V_{C2}	N_{V0} =	N_{V0} = N_{VB}/1000
	-	-	30 ≤ N_{V0} ≤ 160	Pass? **N/A**

Validation and Control Procedures

Experimental Conditions Control (A)	V_{C1}	V_{C2}	A = 153.0	Pass? **Yes**
	155	151	A ≥ 0.5 x Nv/10	
Neutralizer Toxicity or Filtration Control (B)	V_{C1}	V_{C2}	B = 178.0	Pass? **Yes**
	180	176	B ≥ 0.5 x N_{VB}/1000 or Nv/10	
Method Validation (C)	V_{C1}	V_{C2}	C = 175.0	Pass? **Yes**
Concentration: 100 %	176	174	C ≥ 0.5 x Nv/10	

Test Procedure

Product Concentration	Contact Time (minutes)	Dilution	V_{C1}	V_{C2}	Na = $\bar{x}$ or $\bar{x}_{wm}$ x 10	lg Na	lg R = lg N_0 - lg Na
100 %	2	10^0	<14	<14	<1.40E+02	<2.15	>5.44
		10^{-1}	-	-			
		10^{-2}	-	-			
		10^{-3}	-	-			
		10^0					
		10^{-1}					
		10^{-2}					
		10^{-3}					
		10^0					
		10^{-1}					
		10^{-2}					
		10^{-3}					

Raw Data of Colony Count

	N $^{-6}$		N $^{-7}$		N_V		N_{VB}		A		B		C	
V_{C1}	>330	>330	20	18	78	70	-	-	80	75	92	88	91	85
V_{C2}	>330	>330	21	19	77	75	-	-	77	74	90	86	88	86

Product Concentration	Contact Time (minutes)	Na^0		Na^{-1}		Na^{-2}		Na^{-3}	
		V_{C1}	V_{C2}	V_{C1}	V_{C2}	V_{C1}	V_{C2}	V_{C1}	V_{C2}
100 %	2	0	0	-	-	-	-	-	-
		0	0	-	-	-	-	-	-

Appendix 2 Raw data

Test Method	EN 1276:2019				
Product	Hand Cleaning Wipes NO		**Batch No.**	14122023_1	
Product Diluent	Not applicable; ready-to-use product		**Lab No.**	VX-246-24-0001	
Test Organism	*Staphylococcus aureus ATCC 6538*				
Interfering Substance	0.30 g/L bovine albumin solution				
Test Temperature (°C)	20	**Incubation Temperature (°C)**		36	
Neutralizer or Rinsing Liquid	30.00 g/L Tween 80, 30.00 g/L Saponin, 3.00 g/L Lecithin, 1.00 g/L Histidine, 1.00 g/L Cysteine in Tryptone Soya Broth				
Inactivation Method	Dilution-neutralization		**Plating Method**	Pour plate	
Test Date	21/02/2024	**Analyzed By**	AWA	**Verified By**	ANE

Test and Validation Suspension

	N	V_{C1}	V_{C2}		
Test Suspension (N)	10^{-6}	>330	>330	$\bar{x}_{wm}$ = N = 4.70E+08	N_0 = N/10
	10^{-7}	48	46	lg N_0 = 7.67	7.17 ≤ lg N_0 ≤ 7.70
				Pass? **Yes**	

	V_{C1}	V_{C2}		
Validation Suspension (N_V)	155	153	N_{V0} = 154.0	N_{V0} = N_V/10
			30 ≤ N_{V0} ≤ 160	Pass? **Yes**
Validation Suspension (N_{VB})	V_{C1}	V_{C2}	N_{V0} =	N_{V0} = N_{VB}/1000
	-	-	30 ≤ N_{V0} ≤ 160	Pass? **N/A**

Validation and Control Procedures

	V_{C1}	V_{C2}		
Experimental Conditions Control (A)	227	223	A = 225.0	Pass? **Yes**
			A ≥ 0.5 x Nv/10	
Neutralizer Toxicity or Filtration Control (B)	V_{C1}	V_{C2}	B = 201.0	Pass? **Yes**
	204	198	B ≥ 0.5 x N_{VB}/1000 or Nv/10	
Method Validation (C)	V_{C1}	V_{C2}	C = 193.0	Pass? **Yes**
Concentration: 100 %	194	192	C ≥ 0.5 x Nv/10	

Test Procedure

Product Concentration	Contact Time (minutes)	Dilution	V_{C1}	V_{C2}	Na = $\bar{x}$ or $\bar{x}_{wm}$ x 10	lg Na	lg R = lg N_0 - lg Na
100 %	2	10^0	<14	<14	<1.40E+02	<2.15	>5.53
		10^{-1}	-	-			
		10^{-2}	-	-			
		10^{-3}	-	-			
		10^0					
		10^{-1}					
		10^{-2}					
		10^{-3}					
		10^0					
		10^{-1}					
		10^{-2}					
		10^{-3}					

Raw Data of Colony Count

	N^{-6}	N^{-7}	N_V	N_{VB}	A	B	C
V_{C1}	>330	48	155	-	227	204	194
V_{C2}	>330	46	153	-	223	198	192

Product Concentration	Contact Time (minutes)	Na^0		Na^{-1}		Na^{-2}		Na^{-3}	
		V_{C1}	V_{C2}	V_{C1}	V_{C2}	V_{C1}	V_{C2}	V_{C1}	V_{C2}
100 %	2	0	0	-	-	-	-	-	-

Note

cfu: Colony forming units

V_C: Number of cfu counted per 1.0 ml sample

$\overline{x}$: Average V_{C1} and V_{C2} values

N: Number of cfu per ml in the test suspension

N_0: Number of cfu per ml at the beginning of the contact time

N_{V0}: Number of cfu per ml in the mixtures A and C at the beginning of the contact time

N_{VB}: Number of cfu per ml in the mixture B at the beginning of the contact time

N_a: Number of survivors per ml in the test mixture at the end of the contact time and before neutralization

A: Number of cfu per ml in the experimental conditions control

B: Number of cfu per ml in the neutralizer toxicity control

C: Number of cfu per ml in the dilution-neutralization method validation

Appendix 3 Summary of test description

1. Materials and reagents

1.1 Tryptone Soya Agar (TSA, Oxoid, catalogue no. CM0131)

1.2 Tryptone, pancreatic digest of casein (Oxoid, catalogue no. LP0042)

1.3 Sodium chloride (Merck, catalogue no. 1.06404.0500)

1.4 Magnesium chloride ($MgCl_2$, Acros Organics, catalogue no. AC223211000)

1.5 Calcium chloride ($CaCl_2$, R&M Chemicals, catalogue no. 9924-00)

1.6 Sodium bicarbonate ($NaHCO_3$, Fisher Chemical, catalogue no. 10152780)

1.7 Bovine albumin fraction V (Merck, catalogue no. 1.12018.0100)

1.8 Neutralizer

1.8.1 Tween 80 (Fisher Chemical, catalogue no. 10498800)

1.8.2 Saponin (Nacalai Tesque, catalogue no. 30502-55)

1.8.3 Lecithin (Nacalai Tesque, catalogue no. 20335-65)

1.8.4 L-Histidine (Fisher Scientific, catalogue no. BP382-100)

1.8.5 L-Cysteine (Merck, catalogue no. 1.02838.0100)

1.8.6 Tryptone Soya Broth (TSB, Oxoid, catalogue no. CM0129)

2. Apparatus and glassware

2.1 Autoclave (TOMY, model SX500)

2.2 Water baths (Memmert, model WNB 29)

2.3 Incubator (Binder, model BD 260)

2.4 pH-meter (Ohaus, model 3100 Meter with ST310)

2.5 Vortex® mixer (Biosan model Biosan V-1 Plus)

2.6 Petri dishes (Wanpow Plastic)

3. Test procedure

3.1 Test Na – Determination of bactericidal concentrations

3.1.1 Pipette 1.0 ml of interfering substance into a tube.

3.1.2 Add 1.0 ml of the test suspension.

3.1.3 Start the stopwatch immediately, mix and place the tube in a water bath controlled at the chosen test temperature θ for 2 minutes ± 10 seconds.

3.1.4 At the end of this time, add 8.0 ml of the product test solution.

3.1.5 Restart the stopwatch at the beginning of the addition.

3.1.6 Mix and place the tube in a water bath controlled at θ for the chosen contact time *t*.

3.1.7 Just before the end of *t*, mix again.

3.1.8 At the end of *t*, take 1.0 ml sample of the test mixture Na and transfer into a tube containing 8.0 ml neutralizer and 1.0 ml water.

3.1.9 Mix and place in a water bath controlled at 20 °C ± 1 °C.

3.1.10 After a neutralization time of 5 minutes ± 10 seconds, mix and immediately take a sample of 1.0 ml of the neutralized test mixture Na (containing neutralizer, product test solution, interfering substance, and test suspension) in duplicate and inoculate using the pour plate or spread plate technique.

3.1.11 When using the pour plate technique, pipette each 1.0 ml sample into separate Petri dishes and add 15 ml to 20 ml of melted TSA, cooled to 45 °C ± 1 °C.

3.1.12 When using the spread plate technique, spread each 1.0 ml sample – divided into portions of approximately equal size – on an appropriate number (at least two) of surface dried plates containing TSA.

3.1.13 Incubate the plates for 20 to 24 hours at 36 °C ± 1 °C.

3.1.14 Discard any plates which is not countable (for any reason). Count the plates and determine the number of cfu. Incubate the plates for a further 20 to 24 hours.

3.1.15 Do not recount plates which no longer show well separated colonies. Recount the remaining plates. If the number has increased, use only the higher number for further evaluation.

3.1.16 Note the exact number of colonies for each plate but record >330 for any counts higher than 330 and determine the V_C-values.

3.1.17 Perform the procedure using the other product test solutions at the same time.

3.1.18 Perform the procedure applying the other obligatory and – if appropriate – other additional experimental conditions.

3.2 Experimental conditions control A – Validation of the selected experimental conditions and/or verification of the absence of any lethal effect in the test conditions

3.2.1 Pipette 1.0 ml of interfering substance into a tube.

3.2.2 Add 1.0 ml of the validation suspension.

3.2.3 Start the stopwatch immediately, mix, and place the tube in a water bath controlled at θ for 2 minutes ± 10 seconds.

3.2.4 At the end of this time, add 8.0 ml of hard water. In the case of ready-to-use products: water instead of hard water.

3.2.5 Restart the stopwatch at the beginning of the addition.

3.2.6 Mix and place the tube in a water bath controlled at θ for *t*.

3.2.7 Just before the end of *t*, mix again.

3.2.8 At the end of *t*, take a sample of 1.0 ml of this mixture A in duplicate and inoculate using the pour plate or the spread plate technique.

3.2.9 Calculate the numbers of cfu/ml in the validation mixture A.

3.2.10 Verify according to Section 3.5.

3.3 Neutralizer control B – Verification of the absence of toxicity of the neutralizer

3.3.1 Pipette 8.0 ml of the neutralizer used in the test and 1.0 ml of water into a tube.

3.3.2 Add 1.0 ml of the validation suspension.

3.3.3 Start the stopwatch at the beginning of the addition and mix.

3.3.4 Place the tube in a water bath controlled at 20 °C ± 1 °C for 5 minutes ± 10 seconds.

3.3.5 Just before the end of this time, mix.

3.3.6 At the end of this time, take a sample of 1.0 ml of this mixture B in duplicate and inoculate using the pour plate or the spread plate technique.

3.3.7 Calculate the numbers of cfu/ml in the validation mixture B.

3.3.8 Verify according to Section 3.5.

3.4 Method validation C – Dilution-neutralization validation

3.4.1 Pipette 1.0 ml of interfering substance into a tube.

3.4.2 Add 1.0 ml of the diluent.

3.4.3 Start the stopwatch, add 8.0 ml of the product test solution only of the highest concentration used in the test.

3.4.4 Mix and place the tube in a water bath controlled at θ for *t*.

3.4.5 Just before the end of *t*, mix again.

3.4.6 At the end of *t*, transfer 1.0 ml of the mixture into a tube containing 8.0 ml of neutralizer.

3.4.7 Restart the stopwatch at the beginning of the addition.

3.4.8 Mix and place the tube in a water bath controlled at 20 °C ± 1 °C for 5 minutes ± 10 seconds.

3.4.9 Add 1.0 ml of the validation suspension.

3.4.10 Start the stopwatch at the beginning of the addition and mix.

3.4.11 Place the tube in a water bath controlled at 20 °C ± 1 °C for 30 minutes ± 1 minute.

3.4.12 Just before the end of this time, mix again.

3.4.13 At the end of this time, take a sample of 1.0 ml of the mixture C in duplicate and inoculate using the pour plate or the spread plate technique.

3.4.14 Calculate the numbers of cfu/ml in the validation mixture C.

3.4.15 Verify according to Section 3.5.

3.5 Basic limits

3.5.1 N is between 1.5 x 10^8 and 5.0 x 10^8 ($8.17 \leq \lg N \leq 8.70$)

3.5.2 N_0 is between 1.5 x 10^7 and 5.0 x 10^7 ($7.17 \leq \lg N_0 \leq 7.70$)

3.5.3 N_{V0} is between 30 and 160 (3.0 x 10^1 and 1.6 x 10^2)

3.5.4 N_V is between 3.0 x 10^2 and 1.6 x 10^3

3.5.5 A, B, C are equal to or greater than 0.5 x N_{V0}

3.5.6 Control of weighted mean counts: quotient is not lower than 5 and not higher than 15

4. Literature

4.1 EN 1276:2019 (E): Chemical disinfectants and antiseptics – Quantitative suspension test for the evaluation of bactericidal activity of chemical disinfectants and antiseptics used in food, industrial, domestic and institutional areas – Test method and requirements (phase 2, step 1)

4.2 EN 14885:2018 (E): Chemical disinfectants and antiseptics – Application of European Standards for chemical disinfectants and antiseptics

4.3 EN 12353:2013 (E): Chemical disinfectants and antiseptics – Preservation of test organisms used for the determination of bactericidal (including Legionella), mycobactericidal, sporicidal, fungicidal and virucidal (including bacteriophages) activity

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