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Test Report No.: TR-23-0144

Determination of the Bactericidal Activity of **GLOBACID AF MED** according to EN 17387:2021

Test Method
EN 17387:2021
Chemical disinfectants and antiseptics – Quantitative test for the evaluation of bactericidal and yeasticidal and/or fungicidal activity of chemical disinfectants in the medical area on non-porous surfaces without mechanical action – Test method and requirements (Phase 2, step 2)

Client
Goodpoint Chemicals
Urda tee 2/1
Jälgimäe 76404
Estonia

Testing Laboratory
TECOLAB Sdn. Bhd.
J-2-6, Pusat Komersial Jalan Kuching
No. 115, Jalan Kepayang, Off Jalan Kuching
51200 Kuala Lumpur
Malaysia

Kuala Lumpur, 8 March 2023

Dr Marven Lee Cheng Shoou
Managing Director

IDENTIFICATION OF TESTING LABORATORY

TECOLAB Sdn. Bhd.
J-2-6, Pusat Komersial Jalan Kuching
No. 115, Jalan Kepayang, Off Jalan Kuching
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IDENTIFICATION OF CLIENT

Goodpoint Chemicals
Urda tee 2/1
Jälgimäe 76404
Estonia

IDENTIFICATION OF TEST ITEM

Test item name:	Globacid AF Med
Lab ID:	G007-23-001
Batch no.:	102022L
Expiry date:	October 2025
Manufacturer:	Goodpoint Chemicals
Receipt date:	28 February 2023
Storage conditions:	Room temperature away from sunlight
Product diluent recommended by manufacturer:	Not specified
Active substances:	60% Propan-2-ol (CAS: 67-63-0) 15% Ethanol (CAS: 64-17-5)
Product appearance:	Clear, colourless liquid

TEST METHOD & VALIDATION

Test method:	EN 17387:2021 Chemical disinfectants and antiseptics – Quantitative test for the evaluation of bactericidal and yeasticidal and/or fungicidal activity of chemical disinfectants in the medical area on non-porous surfaces without mechanical action – Test method and requirements (Phase 2, step 2)
Inactivation method:	Dilution-neutralization method
Inactivator:	30 g/L Tween 80 30 g/L Saponin 3 g/L Lecithin
Test carrier:	Stainless steel disc 304, grade 2B, 2 cm in diameter, 1.5 mm gauge

EXPERIMENTAL CONDITIONS

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Date of test:	2 March – 6 March 2023
Product diluent:	Distilled water
Concentration / contact time:	100% / 3 minutes ± 10 seconds
Test temperature:	(20 ± 1) °C
Interfering substance:	Clean condition (0.3 g/L bovine serum albumin)
Test organism:	*Enterococcus hirae* ATCC 10541 *Pseudomonas aeruginosa* ATCC 15442 *Staphylococcus aureus* ATCC 6538
Incubation temperature:	(37 ± 1) °C
Incubation period:	24 hours
Appearance of the product dilutions:	Clear, colourless liquid
Stability and appearance of product dilutions during test:	Homogenous without any precipitate

CONTROLS AND VALIDATION

Test Organism	Water Control	Neutralizer Control	Method Validation
E. hirae ATCC 10541	N_C: 8.86 x 10^7 lg N_C: 7.95	NC: 8.59 x 10^7 lg NC: 7.93	NT: 9.91 x 10^7 lg NT: 8.00
P. aeruginosa ATCC 15442	N_C: 4.35 x 10^7 lg N_C: 7.64	NC: 5.25 x 10^7 lg NC: 7.72	NT: 6.10 x 10^7 lg NT: 7.79
S. aureus ATCC 6538	N_C: 1.30 x 10^8 lg N_C: 8.11	NC: 1.25 x 10^8 lg NC: 8.10	NC: 1.17 x 10^8 lg NC: 8.07

The control and validation tests Nc, NC, and NT were within the basic limits:

- lg Nc must be sufficiently high in order to demonstrate a 5-log reduction for bacteria and a 4-log reduction for fungi,
- lg NC – lg N_C must not be greater than ± 0.30 log to verify the absence of neutralizer toxicity, and
- lg NT – lg N_C must not be greater than ± 0.30 log to validate the dilution-neutralization method.

TEST RESULTS

For each product concentration and contact time, the log reduction (lg R) is calculated using the formula lg R = lg N_C – lg Nd, in which:

- N_C is the number of cells per mL in the water control at end of the contact time and before neutralization, and
- Nd is the number of cells per mL in the test mixture at the end of the contact time and before neutralization.

Test organism: *Enterococcus hirae* ATCC 10541

Test suspension, N	N: 1.18×10^8 lg N: 8.07
Water control, N_C	N_C: 8.86×10^7 lg N_C: 7.95

Concentration / Contact Time	**Test, Nd**	**Reduction, lg R = lg N_C – lg Nd**
100% / 3 minutes	Nd: 1.75×10^2 lg Nd: 2.24	lg R: 5.70 ± 0.11 %R: 99.9998%

Test organism: *Pseudomonas aeruginosa* ATCC 15442

Test suspension, N	N: 1.21×10^8 lg N: 8.08
Water control, N_C	N_C: 4.35×10^7 lg N_C: 7.64

Concentration / Contact Time	**Test, Nd**	**Reduction, lg R = lg N_C – lg Nd**
100% / 3 minutes	Nd: $<1.40 \times 10^2$ lg Nd: <2.15	lg R: >5.49 ± 0.11 %R: >99.9997%

Test organism: *Staphylococcus aureus* ATCC 6538

Test suspension, N	N: 1.10×10^8 lg N: 8.04
Water control, N_C	N_C: 1.30×10^8 lg N_C: 8.11

Concentration / Contact Time	**Test, Nd**	**Reduction, lg R = lg N_C – lg Nd**
100% / 3 minutes	Nd: $<1.40 \times 10^2$ lg Nd: <2.15	lg R: >5.97 ± 0.11 %R: >99.9998%

CONCLUSION

The test item achieved a reduction of ≥5.00 log against the test organisms *Enterococcus hirae* ATCC 10541, *Pseudomonas aeruginosa* ATCC 15442, and *Staphylococcus aureus* ATCC 6538.

Therefore, **Globacid AF Med** has demonstrated a bactericidal activity according to EN 17387:2021 under the following conditions:

Concentration	Contact Time	Test Temperature	Soiling
100%	3 minutes	20 °C	Clean condition

Kuala Lumpur, 8 March 2023

Norazzira Zulkharnain
Microbiologist

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EXPERT OPINION

This expert opinion is based on the test report TR-23-0144 dated 8 March 2023. Opinions and interpretations expressed herein are outside the scope of the Laboratory Accreditation Scheme of Malaysia (SAMM).

The product **Globacid AF Med** was tested according to EN 17387:2021 against *Enterococcus hirae* ATCC 10541, *Pseudomonas aeruginosa* ATCC 15442, and *Staphylococcus aureus* ATCC 6538. These organisms are the minimum test bacteria and they have been chosen as representative species taking into account their relative resistance, relevance to practical use, handling properties, and microbiological safety.

Bactericidal activity is defined as a capability of a product or active substance to produce a reduction in the number of viable bacterial cell of relevant test organisms under defined conditions, respectively. According to EN 17387, a surface disinfectant without mechanical action for non-porous surfaces is considered to possess bactericidal and fungicidal activities if it demonstrates a reduction of ≥5.00 log and a reduction of ≥4.00 log, respectively, against the minimum spectrum of test organisms within 60 minutes when tested at 4 to 40 °C under clean (0.3 g/L bovine serum albumin) or dirty (3.0 g/L bovine serum albumin and 3.0 ml/L sheep erythrocytes) condition. A yeasticidal activity is demonstrated if the required log reduction of ≥4.00 is achieved against the minimum spectrum of yeast only.

When tested under the following conditions, **Globacid AF Med** achieved a reduction of ≥5.00 log against *Enterococcus hirae* ATCC 10541, *Pseudomonas aeruginosa* ATCC 15442, and *Staphylococcus aureus* ATCC 6538:

Concentration	Contact Time	Test Temperature	Soiling
100%	3 minutes	20 °C	Clean condition

Therefore, **Globacid AF Med** has demonstrated a bactericidal activity conforming to EN 17387:2021 under the aforementioned conditions.

Kuala Lumpur, 8 March 2023

Dr Marven Lee Cheng Shoou
Managing Director

INFORMATION ON MEASUREMENT UNCERTAINTY & DECISION RULE

The statement of conformity given by EN 17387:2021 states that the test item shall be considered to have passed EN 17387 if it demonstrates ≥5.00 log reduction for bacteria and ≥4.00 log reduction for fungi/yeast under the defined conditions.

The laboratory employs the simple acceptance decision rule to account for the measurement uncertainty when stating the statement of conformity. The measurement uncertainty and conformance probability are shown in the raw data and are summarized as follows:

Test Organism	Concentration / Contact Time	Log Reduction	Conformance	Conformance Probability†
E. hirae ATCC 10541	100% / 3 minutes	5.70 ± 0.11	Yes	<0.001% chance of false acceptance
P. aeruginosa ATCC 15442	100% / 3 minutes	>5.49 ± 0.11	Yes	<0.001% chance of false acceptance
S. aureus ATCC 6538	100% / 3 minutes	>5.97 ± 0.11	Yes	<0.001% chance of false acceptance

† The conformance probability follows a normal distribution. Therefore, the percentage of conformance can never be zero or 100% due to the asymptotic tails.

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RAW DATA

Test Method:	EN 17387:2021		
Product Name:	Globacid AF Med	Batch No.:	102022L
Product Diluent:	Distilled water	Lab ID:	G007-23-001
Appearance of Product Dilutions:	Clear, colourless solution		
Inactivation:	Dilution-neutralization	Test Temperature (°C):	20
Carrier Drying Time (min):	13		
Neutralizer:	30 g/L Tween 80, 30 g/L Saponin, 3 g/l Lecithin		
Interfering Substance:	0.3 g/L bovine serum albumin		
Test Organism:	Enterococcus hirae ATCC 10541	Plating:	Pour plate
Incubation Temperature (°C):	37	Passing Criteria (lg):	5.00
Measurement Uncertainty (±):	0.11		
Testing Period:	02/03/2023	Tested By:	AZZ
Verified By:	CSE		

Validation & Controls

	N_C	V_{C1}	V_{C2}	
Water Control (N_C)	10^{-4}	>330	>330	$\bar{x}_{wm} \times 10 = N_C =$ 8.86E+07
	10^{-5}	84	81	lg N_C = 7.95
	10^{-6}	14	16	Nts = >330 cfu/mL
	10^{-7}	<14	<14	Limit: $N_C \geq 7.15$

	NC	V_{C1}	V_{C2}	
Neutralizer Control (NC)	10^{-4}	>330	>330	$\bar{x}_{wm} \times 10 = NC =$ 8.59E+07
	10^{-5}	77	80	lg NC = 7.93
	10^{-6}	18	14	Limit: $\lvert lg\ NC - lg\ N_C \rvert \leq 0.3$
	10^{-7}	<14	<14	

	NT	V_{C1}	V_{C2}	
Method Validation (NT)	10^{-4}	>330	>330	$\bar{x}_{wm} \times 10 = NT =$ 9.91E+07
	10^{-5}	86	95	lg NT = 8.00
	10^{-6}	20	17	Limit: $\lvert lg\ NT - lg\ N_C \rvert \leq 0.3$
Conc.: 100%	10^{-7}	<14	<14	

Test Suspension & Procedure

	N	V_{C1}	V_{C2}	
Test Suspension (N)	10^{-7}	>330	>330	$\bar{x}_{wm} \times 0.025 = N =$ 1.18E+08
	10^{-8}	43	51	lg N = 8.07
				Limit: 7.57 ≤ lg N ≤ 8.10

Product Concentration	Contact Time	Dilution	V_{C1}	V_{C2}	Nd = $\bar{x}$ or $\bar{x}_{wm} \times 10$	lg Nd	lg R = lg N_C - lg Nd	Nts	Conformance Probability
100%	3 min	10^{0}	19	16	1.75E+02	2.24	5.70 ± 0.11	0	>99.999%
		10^{-1}	<14	<14					
		10^{-2}	<14	<14			99.9998%		
		10^{-3}	<14	<14					
		10^{0}							
		10^{-1}							
		10^{-2}							
		10^{-3}							
		10^{0}							
		10^{-1}							
		10^{-2}							
		10^{-3}							

Raw Data of Colony Count

	N_C $^{-4}$	N_C $^{-5}$	N_C $^{-6}$	N_C $^{-7}$	NC $^{-4}$	NC $^{-5}$	NC $^{-6}$	NC $^{-7}$	NT $^{-4}$	NT $^{-5}$	NT $^{-6}$	NT $^{-7}$	N $^{-7}$	N $^{-8}$
V_{C1}	>330	84	14	1	>330	77	18	1	>330	86	20	2	>330	43
V_{C2}	>330	81	16	0	>330	80	14	0	>330	95	17	1	>330	51

Product Concentration	Contact Time	Nts (cfu/mL)	Nd ^{0}		Nd $^{-1}$		Nd $^{-2}$		Nd $^{-3}$	
			V_{C1}	V_{C2}	V_{C1}	V_{C2}	V_{C1}	V_{C2}	V_{C1}	V_{C2}
100%	3 min	0	19	16	0	0	0	0	0	0

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RAW DATA

Test Method:	EN 17387:2021		
Product Name:	Globacid AF Med	Batch No.:	102022L
Product Diluent:	Distilled water	Lab ID:	G007-23-001
Appearance of Product Dilutions:	Clear, colourless solution		
Inactivation:	Dilution-neutralization	Test Temperature (°C):	20
Carrier Drying Time (min):	12		
Neutralizer:	30 g/L Tween 80, 30 g/L Saponin, 3 g/L Lecithin		
Interfering Substance:	0.3 g/L bovine serum albumin		
Test Organism:	Pseudomonas aeruginosa ATCC 15442	Plating:	Spread plate
Incubation Temperature (°C):	37	Passing Criteria (lg):	5.00
Measurement Uncertainty (±):	0.11		
Testing Period:	06/03/2023	Tested By:	SCCY
Verified By:	CSE		

Validation & Controls

	N_C	V_{C1}	V_{C2}	
Water Control (N_C)	10^{-4}	>660	>660	$\bar{x}_{wm}$ x 10 = N_C = 4.35E+07
	10^{-5}	41	46	lg N_C = 7.64
	10^{-6}	<14	<14	Nts = >330 cfu/mL
	10^{-7}	<14	<14	Limit: $N_C \geq 7.15$

	NC	V_{C1}	V_{C2}	
Neutralizer Control (NC)	10^{-4}	>660	>660	$\bar{x}_{wm}$ x 10 = NC = 5.25E+07
	10^{-5}	55	50	lg NC = 7.72
	10^{-6}	<14	<14	Limit: $\lvert lg\ NC - lg\ N_C \rvert \leq 0.3$
	10^{-7}	<14	<14	

	NT	V_{C1}	V_{C2}	
Method Validation (NT)	10^{-4}	>660	>660	$\bar{x}_{wm}$ x 10 = NT = 6.10E+07
	10^{-5}	62	60	lg NT = 7.79
	10^{-6}	<14	<14	Limit: $\lvert lg\ NT - lg\ N_C \rvert \leq 0.3$
Conc.: 100%	10^{-7}	<14	<14	

Test Suspension & Procedure

	N	V_{C1}	V_{C2}	
Test Suspension (N)	10^{-7}	475	492	$\bar{x}_{wm}$ x 0.025 = N = 1.21E+08
	10^{-8}	44	52	lg N = 8.08
				Limit: 7.57 ≤ lg N ≤ 8.10

Product Concentration	Contact Time	Dilution	V_{C1}	V_{C2}	Nd = $\bar{x}$ or $\bar{x}_{wm}$ x 10	lg Nd	lg R = lg N_C - lg Nd	Nts	Conformance Probability
100%	3 min	10^{0}	<14	<14	<1.40E+02	<2.15	>5.49 ± 0.11	0	>99.999%
		10^{-1}	<14	<14					
		10^{-2}	<14	<14			>99.9997%		
		10^{-3}	<14	<14					
		10^{0}							
		10^{-1}							
		10^{-2}							
		10^{-3}							
		10^{0}							
		10^{-1}							
		10^{-2}							
		10^{-3}							

Raw Data of Colony Count

	N_C $^{-4}$	N_C $^{-5}$	N_C $^{-6}$	N_C $^{-7}$	NC $^{-4}$	NC $^{-5}$	NC $^{-6}$	NC $^{-7}$	NT $^{-4}$	NT $^{-5}$	NT $^{-6}$	NT $^{-7}$	N $^{-7}$	N $^{-8}$
V_{C1}	>330	20	2	0	>330	25	2	0	>330	30	3	0	230	23
	>330	21	0	0	>330	30	3	0	>330	32	3	0	245	21
V_{C2}	>330	24	1	0	>330	25	2	0	>330	30	3	0	250	30
	>330	22	0	0	>330	25	2	0	>330	30	3	0	242	22

Product Concentration	Contact Time	Nts (cfu/mL)	Nd ^{0}		Nd $^{-1}$		Nd $^{-2}$		Nd $^{-3}$	
			V_{C1}	V_{C2}	V_{C1}	V_{C2}	V_{C1}	V_{C2}	V_{C1}	V_{C2}
100%	3 min	0	2	0	0	0	0	0	0	0
			3	0	0	0	0	0	0	0

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RAW DATA

Test Method:	EN 17387:2021				
Product Name:	Globacid AF Med			Batch No.:	102022L
Product Diluent:	Distilled water			Lab ID:	G007-23-001
Appearance of Product Dilutions:	Clear, colourless solution				
Inactivation:	Dilution-neutralization	Test Temperature (°C):	20	Carrier Drying Time (min):	13
Neutralizer:	30 g/L Tween 80, 30 g/L Saponin, 3 g/l Lecithin				
Interfering Substance:	0.3 g/L bovine serum albumin				
Test Organism:	Staphylococcus aureus ATCC 6538			Plating:	Pour plate
Incubation Temperature (°C):	37	Passing Criteria (lg):	5.00	Measurement Uncertainty (±):	0.11
Testing Period:	02/03/2023	Tested By:	NII	Verified By:	CSE

Validation & Controls

	N_C	V_{C1}	V_{C2}	
Water Control (N_C)	10^{-4}	>330	>330	$\bar{x}_{wm} \times 10 = N_C =$ 1.30E+08
	10^{-5}	133	118	lg N_C = 8.11
	10^{-6}	18	16	Nts = >330 cfu/mL
	10^{-7}	<14	<14	Limit: $N_C \geq 7.15$

	NC	V_{C1}	V_{C2}	
Neutralizer Control (NC)	10^{-4}	>330	>330	$\bar{x}_{wm} \times 10 = NC =$ 1.25E+08
	10^{-5}	112	123	lg NC = 8.10
	10^{-6}	18	22	Limit: $\lvert lg\ NC - lg\ N_C \rvert \leq 0.3$
	10^{-7}	<14	<14	

	NT	V_{C1}	V_{C2}	
Method Validation (NT)	10^{-4}	>330	>330	$\bar{x}_{wm} \times 10 = NT =$ 1.17E+08
	10^{-5}	115	116	lg NT = 8.07
	10^{-6}	<14	15	Limit: $\lvert lg\ NT - lg\ N_C \rvert \leq 0.3$
Conc.: 100%	10^{-7}	<14	<14	

Test Suspension & Procedure

	N	V_{C1}	V_{C2}	
Test Suspension (N)	10^{-7}	>330	>330	$\bar{x}_{wm} \times 0.025 = N =$ 1.10E+08
	10^{-8}	49	39	lg N = 8.04
				Limit: 7.57 ≤ lg N ≤ 8.10

Product Concentration	Contact Time	Dilution	V_{C1}	V_{C2}	Nd = $\bar{x}$ or $\bar{x}_{wm} \times 10$	lg Nd	lg R = lg N_C - lg Nd	Nts	Conformance Probability
100%	3 min	10^{0}	<14	<14	<1.40E+02	<2.15	>5.97 ± 0.11	0	>99.999%
		10^{-1}	<14	<14					
		10^{-2}	<14	<14			>99.9999%		
		10^{-3}	<14	<14					
		10^{0}							
		10^{-1}							
		10^{-2}							
		10^{-3}							
		10^{0}							
		10^{-1}							
		10^{-2}							
		10^{-3}							

Raw Data of Colony Count

	N_C $^{-4}$	N_C $^{-5}$	N_C $^{-6}$	N_C $^{-7}$	NC $^{-4}$	NC $^{-5}$	NC $^{-6}$	NC $^{-7}$	NT $^{-4}$	NT $^{-5}$	NT $^{-6}$	NT $^{-7}$	N $^{-7}$	N $^{-8}$
V_{C1}	>330	133	18	2	>330	112	18	5	>330	115	13	1	>330	49
V_{C2}	>330	118	16	3	>330	123	22	1	>330	116	15	0	>330	39

Product Concentration	Contact Time	Nts (cfu/mL)	Nd ^{0}		Nd $^{-1}$		Nd $^{-2}$		Nd $^{-3}$	
			V_{C1}	V_{C2}	V_{C1}	V_{C2}	V_{C1}	V_{C2}	V_{C1}	V_{C2}
100%	3 min	0	0	0	0	0	0	0	0	0

TEST PROCEDURE

1. Test *Nd*: Determination of Bactericidal and/or Fungicidal Concentrations

   1.1 To prepare the microbial test suspension, 1.0 mL of the interfering substance was pipetted into a tube. 1.0 mL of the test suspension *N* (1.5 – 5.0 x 10^9 cfu/mL for bacteria; 1.5 – 5.0 x 10^8 cfu/mL for fungi) was added to the tube.

   1.2 The stopwatch was started immediately and the tube was mixed and placed in a water bath controlled at the test temperature (θ ± 1) °C for 2 minutes ± 10 seconds.

   1.3 The test surface was placed in a sterile Petri dish, ensuring that the dish was in a horizontal position. The test surface was prepared by inoculating with 0.05 mL of the microbial test suspension onto each test surface. The surface was dried at room temperature or at 37 °C until they are visibly dry. The drying time should not exceed 60 minutes. The test surfaces were equilibrated with the chosen test temperature θ.

   1.4 0.1 mL of product test solution was pipetted onto separate dried test surfaces ensuring that the dried inoculum was totally covered by the test product. The surface was placed in a temperature-controlled cabinet at the chosen test temperature θ and contact time *t*.

   1.5 At the end of *t*, each of the test surface *Nd* was transferred to a separate container containing 10 mL of neutralizer together with sufficient glass beads to support the test surface. The surface was placed with the inoculated surface downwards in contact with the beads. The container was shaken for a minimum of 1 minute. The shaking should be sufficiently vigorous to ensure that the test surface moved constantly over the beads.

   1.6 After a neutralization time of 5 minutes ± 10 seconds, a series of 10-fold dilutions were prepared from 10^{-1} to 10^{-2} of the neutralized mixture in diluent. 1.0 mL sample of the neutralized mixture and each of the dilutions were taken in duplicate and inoculated using pour plate or spread plate technique.

   1.7 The test surface *Nts* was recovered by letting the neutralizer drain off and rinsing with 10 mL of distilled water. The test surface was transferred to a Petri dish containing 10 mL of solidified agar, with the test side facing upwards. 10 mL of melted agar was added onto the plated test surface.

   1.8 The procedure was performed using other product test solutions at the same time.

2. Water Control N_C: Verification of the Absence of Any Lethal Effect in the Experimental Conditions

   2.1 The water control N_C was conducted in parallel with the test *Nd*. The product test solution was substituted with hard water (distilled water in the case of ready-to-use products).

   2.2 After a neutralization time of 5 minutes ± 10 seconds, a series of 10-fold dilutions were prepared. For bacterial strains, 10^{-4} to 10^{-7} dilutions of the neutralized mixture were prepared in diluent and for fungal strains, 10^{-3} to 10^{-6}. 1.0 mL sample of each of the dilutions were taken in duplicate and inoculated using pour plate or spread plate technique.

   2.3 The test surface *Nts* was recovered the same way as the test *Nd*.

3. Neutralizer Control *NC*: Verification of the Absence of Toxicity of the Neutralizer

   3.1 One inoculated test surface was prepared.

   3.2 10 mL of the neutralizer was pipetted into a container with sufficient glass bead. 0.1 mL of hard water (distilled water in the case of ready-to-use products) was added. The mixture was mixed and left in contact for 5 minutes ± 10 seconds at (20 ± 1) °C.

3.3 The inoculated and dried test surface was transferred into the container at the end of neutralization time and placed downwards in contact with the glass bead. The container was shaken for a minimum of 1 minute. The shaking should be sufficiently vigorous to ensure that the test surface moved constantly over the beads.

3.4 For bacterial strains, 10^{-4} to 10^{-7} dilutions of the neutralized mixture were prepared in diluent and for fungal strains, 10^{-3} to 10^{-6}. 1.0 mL sample of each of the dilutions were taken in duplicate and inoculated using pour plate or spread plate technique.

4. Method Validation *NT*: Validation of the Dilution-Neutralization Method

4.1 One inoculated test surface was prepared.

4.2 10 mL of the neutralizer was pipetted into a container with sufficient glass bead. 0.1 mL of the highest product concentration used in the test *Nd* was added. The mixture was mixed and left in contact for 5 minutes ± 10 seconds at (20 ± 1) °C.

4.3 The inoculated and dried test surface was transferred into the container at the end of neutralization time and placed downwards in contact with the glass bead. The container was shaken for a minimum of 1 minute. The shaking should be sufficiently vigorous to ensure that the test surface moved constantly over the beads.

4.4 For bacterial strains, 10^{-4} to 10^{-7} dilutions of the neutralized mixture were prepared in diluent and for fungal strains, 10^{-3} to 10^{-6}. 1.0 mL sample of each of the dilutions were taken in duplicate and inoculated using pour plate or spread plate technique.

5. Incubation and Counting

5.1 For bacterial strains, the plates were incubated for 20 to 24 hours. The plates were counted to determine the number of cfu. Any plates which were not countable for any reason were discarded.

5.2 For fungal strains, the plates were incubated for 18 to 24 hours for yeast, and 42 to 48 hours for mould. Any plates which were not countable for any reason were discarded. The plates were counted to determine the number of cfu. The plates were incubated for a further 18 to 24 hours. Plates that no longer showed well-separated colonies were not recounted. For mould, the plates were incubated for a further 18 to 24 hours, and if necessary, a further 18 to 24 hours, provided the number of countable colonies were increasing.

5.3 The remaining plates were recounted and if the number of colonies had increased, only the higher number was used for further evaluation.

5.4 For each plate, the exact number of colonies were noted but any counts higher than 330 colonies or 165 colonies (for mould) were recorded as '>330' and '>165', respectively.

5.5 All experimental data were reported as V_C values, in which a V_C value is the number of cfu counted per 1.0 mL sample inoculated.

5.6 Only V_C values within the counting limits, i.e., 14 to 330/165 colonies, were taken into account for further calculation, except in the case of *Nd*.