

Final report 2011/1947 AMi

SUSPENSION BASIC BACTERICIDAL EFFECTIVENESS ON VIRES 5

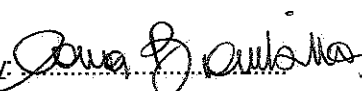
Study Program No: 2011/1947 AM

Contract No: PARA2011040201

Sponsor: VIRES5 BVBA
BREDABAAN 926
2990 WUUSTWEZEL (BELGIUM)

Study monitor: BSL BIOSERVICE SCIENTIFIC LABORATORIES GmbH
BEHRINGSTRASEE 6/8
82152 PLANEGG

Test substance: VIRES 5

Director of the Study: 
(Laura Brambilla)

Released on: Dec 16th 2011

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COMPLIANCE WITH GOOD LABORATORY PRACTICE

I the undersigned declare that the studies described in this report have been conducted under my supervision and in compliance with the following standards of Good Laboratory Practice:

- OECD Series on Principles of Good Laboratory Practice and Compliance Monitoring - OECD principles of Good Laboratory Practice (as revised in 1997) – Environment Directorate – Organisation for Economic Co- Operation and Development, Paris 1998.
- Legislative decree n. 50 of March the 2nd, 2007. Enforcement of Community Directives 2004/9/CE e 2004/10/CE, concerning the inspection and verification of Good Laboratory Practice and the drawing of the legislative, regulatory and administrative dispositions relative to the application of Good Laboratory Practice rules, to the control of their application on the assays performed on the chemical substances (GU n.86 of April the 13th, 2007).
- Decree of the Italian Ministry of Health October the 12th 2010, certification N. 121/2010 authorizing Eurofins Biolab S.r.l. to perform analyses in compliance with the principles of good laboratory practices (<http://www.biolab.it>).

There were no circumstances that may affect the quality or integrity of the study


 Study Director
 (Laura Brambilla)

Dec 16th 2011
 Date

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QUALITY ASSURANCE STATEMENT


The study was assessed for compliance with the approved study program and the Standard Operating Procedures of Eurofins Biolab Srl.

The study and/or the test facility were periodically inspected by the Quality Assurance unit according to the corresponding SOPs. These inspections and audit were carried out by the Quality Assurance unit, personnel independent of staff involved in the study.


The undersigned hereby certifies the dates on which the inspections have been carried out and reported to the Director of the Study and to Eurofins Biolab's S.r.l. Management:

PHASE OF STUDY	DATE OF INSPECTION / REPORTING
Pre-experimental period	//
Experimental period	//
Post-experimental period	//
Documentation: - Study program - Raw data - Final report	November, 29 th 2011 December, 16 th 2011 December, 16 th 2011

This report accurately reflects the raw data.


 QA MANAGER
 (Patrizia Custode)

Dec 16th, 2011
 DATE

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SUMMARY

An assay was conducted on test substance VIRES 5 in order to determine its basic bactericidal effectiveness for the uses for which the product is specifically intended.

The bactericidal effectiveness has been evaluated with the following experimentation:

- **phase 1, basic bactericidal activity suspension test for chemical disinfectants and antiseptics** in which two bacterial strains, *Staphylococcus aureus* ATCC 6538 and *Pseudomonas aeruginosa* ATCC 15442, have been exposed to the test substance in the following conditions:

- final concentrations: 80% (maximum concentration testable) – 25% – 10%
- contact time: 5 minutes
- test temperature: 20°C±1°C

On the basis of the obtained results, in compliance with the assay validity criteria, the test substance VIRES 5 results **BACTERICIDAL** with the concentration of 80% after 5 minutes of contact, in compliance with the provisions of EN 1040:2005.

See *Experimental Report 2011/1947* for more details.

INTRODUCTION

A study was conducted on behalf of VIRES5 BVBA in order to demonstrate the basic bactericidal effectiveness, in accordance with European regulations and Sponsor requirements.

The study was performed at the Test Facility Eurofins Biolab S.r.l. of Vimodrone (MI) – via B. Buozi n. 2 (Italy).

EXPERIMENTATION	START	END	RESEARCHER
Basic bactericidal activity suspension test for chemical disinfectants and antiseptics	Nov 30 th 2011	Dec 02 nd 2011	C. Meroni

In this report:

- The doses are expressed as grams of the test substance for 100 ml of the water (%)
- The number of microorganisms, counted in colony-forming units per milliliter test solution, is expressed as colony-forming units per milliliter (cfu/ml).

TERMS AND DEFINITIONS

Bactericidal: a chemical agent or formulation capable of killing vegetative bacteria under given conditions.

Bactericidal activity: the ability of a product of reducing the number of bacteria under given conditions.

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REFERENCES

EN 1040, December 2005 - Chemical disinfectants and antiseptics – Quantitative suspension test for the evaluation of basic bactericidal activity of chemical disinfectants and antiseptics - Test method and requirements (phase 1).

FILING

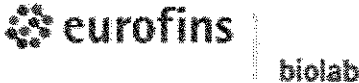
The study program, all raw data are filed in the archives of Eurofins Biolab S.r.L for ten years after the issuing of the final report.

No retained sample will be kept because the Sponsor did not provide stability informations.

At the end of the conservation period, the Sponsor may request an extension of the conservation of all or part of the products for a further period, or their restitution. A suitable agreement shall be drafted in this case.

PROCEDURES

All procedures used during this study are recorded in the Eurofins Biolab S.r.L Procedures Manual.

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TEST SUBSTANCE

The test substance consists of a disinfectant to improve water quality in veterinary field.

Name	VIRES 5
Product description	Purified water with increased OrpV value
Stability	Not provided
Composition	Hypochlorous acid (CAS-No: 7790-92-3) <1% Water (CAS-No: 7732-18-5) 50-100% Other additives <10%

ANALYSED SAMPLE

The analysed sample, representative of the test substance, consists in a transparent colourless liquid contained into a plastic transparent container.

Batch	23107
Code	05231
Manufacture date	Not provided
Expiry date	Not provided
CoA	Not provided
Receiving n.	EUITVI-21224
Receiving date	Nov 17 th 2011
Id number	11.2926-S

The characterisation of the test product is under Sponsor's responsibility

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**Experimental Report 2011/1947 – EVALUATION OF BASIC BACTERICIDAL
 ACTIVITY IN SUSPENSION- DILUTION – NEUTRALIZATION METHOD
 (EN1040:2005)**

EXPERIMENTAL PROCEDURE

1. ASSAY SYSTEM

Microorganisms

The following test strains were used:

<i>Staphylococcus aureus</i>	ATCC 6538
<i>Pseudomonas aeruginosa</i>	ATCC 15442

Conservation

The bacterial strains were kept frozen; before they were used, they were transplanted on TSA slants and kept in a refrigerator at 5°C ±3°C.

Preparation of the bacterial suspensions

The bacterial strains were transplanted on TSA slants twice consecutively and incubated at 37°C ±1°C for 18 hours.

Within two hours from the beginning of the test, the final culture was suspended in the diluent using glass beads, and the suspension was diluted to a concentration of about 1.5×10⁸-5×10⁸ cfu/ml.

The colony number was determined performing the counting.

2. CULTURE MEDIA AND REAGENTS

<i>Tryptone Soya Agar (TSA)</i>	MERCK
--	-------

Diluent

Tryptone, pancreatic digestion of casein	1.0 g	MERCK
NaCl	8.5 g	MERCK
Distilled water q.s. to	1000 ml	

<i>Water for injections (WFI)</i>	EUROSPITAL
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3. EQUIPMENT

Dry sterilization oven	MEMMERT
Steam autoclave	FEDEGARI
Incubator	BINDER
pHmeter	BECKMAN
Vortex stirrer	VELP
Chronometer	GHIARONI
Micropipettes	GILSON
Spectrophotometer	SHIMADZU

4. EXPERIMENTAL DESIGN

Test temperature


The test was conducted at 20°C ±1°C.

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Experimental conditions

The test was performed at the following conditions:

- final concentrations: 80% (maximum concentration testable) – 25% – 10%
- contact time: 5 minutes

The test substance was prepared with a concentration 1.25 times higher than the concentration required to perform the test.

Neutralizer

The following neutraliser was selected:

Lecithin	3 g	MERCK
Polysorbate 80	30 g	MERCK
Sodium Thiosulfate	5 g	MERCK
L-histidine	1 g	MERCK
Saponin	30 g	SIGMA
Tryptone-treated water	q.s. to 1000 ml	

5. EXECUTION OF THE ASSAY

5.1 Preliminary assay

A preliminary assay was conducted prior to the execution of the assay.

The bacterial suspensions had previously been stabilized at the test temperature while the neutralizer and the water had been stabilized at 20°C ± 1°C.

Count of the validation bacterial suspensions

The bacterial suspensions were diluted to a concentration of about 3.0x10² to 1.6x10³ cfu/ml.

This suspension was further diluted using a decimal dilution and the number of colonies was then determined through inclusion in agar after 48 hours' incubation period at 37°C ± 1°C. **Nv** value was then calculated.

Preparation of test substance

The test substance was diluted at the highest concentration tested during the assay.

Validation of the experimental conditions

1 ml of sterile water and 1 ml of bacterial validation suspension containing 3.0x10² to 1.6x10³ cfu/ml were placed in a test tube.

The components were left in contact for 2 minutes; then 8 ml of water were added and left in contact at the temperature adopted during the assay for the longest period to be tested.

At the end of the contact time, the mixture was vortex-stirred and a double count was performed by inclusion in agar.

The number of colony-forming units per ml of the mixture was determined following incubation for 48 hours at 37°C ± 1°C and **A** was calculated.

Validation of the neutralizer non-toxicity

For each test strain, 8 ml of neutraliser, 1 ml of distilled water and 1 ml of bacterial validation suspension (3.0x10² to 1.6x10³ cfu/ml) were mixed in a test tube and left in contact for 5 minutes at 20°C ± 1°C temperature. At the end of the contact time, the mixture was vortex-stirred and a double count was performed by inclusion in agar.


The number of colony-forming units per ml mixture was determined following incubation for 48 hours at 37°C ± 1°C and **B** was calculated.

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Validation of the dilution-neutralization test

For each test strain, 1 ml of sterile water, 1 ml of the diluent and 8 ml of the test substance at the highest tested concentration were mixed in a test tube and left in contact at the temperature adopted during the assay for the longest period chosen. At the end of the contact time, 1 ml of the mixture was transferred into a test tube containing 8 ml of neutraliser and left in contact for 5 minutes. Then 1 ml of bacterial validation suspension 3.0×10^2 to 1.6×10^3 cfu/ml were added and left in contact for 30 minutes at $20^\circ\text{C} \pm 1^\circ\text{C}$. At the end of the contact time, the mixture was vortex-stirred and a double count was performed by inclusion in agar.

The number of colony-forming units per ml mixture was determined following incubation for 48 hours at $37^\circ\text{C} \pm 1^\circ\text{C}$ and C value was calculated.

5.2 Assay

Counting of bacterial suspension

The bacterial suspension showing concentrations in a 1.5×10^8 to 5×10^8 cfu/ml range were diluted up to 10^6 and 10^7 .

A double counting through inclusion in agar was performed. The number of colony-forming units per ml of the suspension was determined following incubation for 48 hours at $37^\circ\text{C} \pm 1^\circ\text{C}$ and N value was calculated.

Assay performing

The assay sample, the bacterial suspensions, the neutraliser agent and the water had previously been stabilised at the test temperature of $20^\circ\text{C} \pm 1^\circ\text{C}$.

For each bacterial strain and for each concentration of the test substance, one test tube containing 1 ml of sterile water and 1 ml of bacterial test suspension showing concentrations in a 1.5×10^8 to 5.0×10^8 cfu/ml range, was prepared at the temperature adopted during the assay.

After 2 minutes of contact, 8 ml of test substance were added and left in contact again for the selected times at the test temperature.

At the end of the contact time (5 minutes), 1 ml of mixture was transferred into a test tube containing 8 ml of neutraliser and 1 ml of distilled water.

After 5 minutes ± 10 sec. of neutralization procedure, the mixture was vortex-stirred and a double count was performed by inclusion in agar.

The number of cfu per plate was determined following incubation for 48 hours at $37^\circ\text{C} \pm 1^\circ\text{C}$, and Na value was then calculated.

6. CALCULATION AND EXPRESSION OF THE RESULTS

Calculation of the viable count (cfu/ml)

The count was performed using the number of colonies counted on both plates.

Only the plates showing a number of colonies included in a 15-300 range were used to perform the result calculation. A deviation of 10% is accepted, so the limits are 14 and 330.

In the assay, where the number of cfu on every plate counted is < 14 , the number of cfu/ml should be recorded as $< 1.4 \times 10^2$.

Where the number of cfu on every plate counted is > 330 , the number of cfu/ml should be recorded as $> 3.3 \times 10^3$.

Test suspension

The calculation of the bacterial count for the suspension test (N) is performed applying the following formula:

$$N(\text{cfu/ml}) = \frac{c}{(n_1 + 0.1n_2)d}$$

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where:

- c = sum of colonies counted on both plates
n₁ = number of counted plates in the lower dilution
n₂ = number of counted plates in the higher dilution
d = dilution factor corresponding to the lower dilution

Assay and preliminary assay

For the calculation of the bacterial count for the assay (**Na**) and for the preliminary assay (**A**, **B**, **C** and **N_v**) is performed applying the following formula:

$$cfu/ml = \frac{C}{n \times V \times d}$$

where:

- C = total of colonies counted on both plates
n = number of counted plates
V = volume used
d = dilution factor corresponding to the relevant dilution

Calculation of vitality reduction

Vitality reduction is expressed in logarithm and was calculated for each organism and test concentration using the following formula:

$$\lg R = \lg N_0 - \lg Na$$

where:


- R = Reduction of vitality
N₀ = N/10
Na = bacterial counting for the test mixture at the end of the contact time

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ASSAY VALIDITY CRITERIA

Verify the following:

N: must be included between 1.5×10^8 and 5.0×10^8 cfu/ml

N_v: must be included between 3.0×10^2 and 1.6×10^3 cfu/ml

A, B, C: must be equal to, or higher than 0.05 times **N_v**

Control Of Weighted Mean Counts: quotient is not lower than 5 and not higher than 15

where:

N: count of cfu/ml in the bacterial test suspension

N_v: count of cfu/ml in the bacterial validation suspension in the preliminary assay

A: count of cfu/ml in the experimental conditions validation

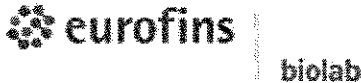
B: count of cfu/ml in the neutraliser toxicity control

C: count of cfu/ml of the neutraliser effectiveness

Weighted Mean Counts: weighted mean of two subsequent dilutions (e.g. "N")

The test substance is considered bactericidal when the bacterial count for each bacterial strain is reduced by at least 5 Log following 5 minutes' contact at 20°C.

The test substance is considered effective against the test microorganisms when the bacterial count for each bacterial strain is reduced by at least 5 Log following the chosen contact time at 20°C.

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RESULTS

Preliminary assay

The N, N_v, A, B, C and the control of weighted mean counts of each bacterial strain comply with the validity criteria. The specific values are shown in Attachment #1.

Assay

The vitality reduction values at the different concentrations tested are shown below and in the Attachment #1:

TEST MICROORGANISMS	CONTACT TIME AND TESTED CONCENTRATIONS		
	5 minutes		
	80%	25%	10%
<i>Staphylococcus aureus</i> ATCC6538	>5.33	>5.33	5.16
<i>Pseudomonas aeruginosa</i> ATCC15442	>5.46	<4.09	<4.09

DEVIATIONS

The study did not undergo deviations compared to the study program.

CONCLUSIONS

On the basis of the obtained results, in compliance with the assay validity criteria, the test substance VIRES 5 results **BACTERICIDAL** with the concentration of 80% after 5 minutes of contact, in compliance with the provisions of EN 1040:2005.


ATTACHMENTS

ATTACHMENT	TITLE	NUMBER OF PAGES
N.1	EXCEL ELABORATION OF EXPERIMENTATION 2011/1947	2

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 eurofins biolab	Prova quantitativa in sospensione per la valutazione dell'attività battericida di base dei disinfettanti chimici e antisettici (Quantitative suspension test for the evaluation of basic bactericidal activity of chemical disinfectants and antiseptics)	
	Norma (Standard): EN 1040:2005-phase1/step1	
	Pagina 1 di 2 (page 1 of 2)	
Mod. PS/MIC/001.E Rev.3		

ID. studio (ID. Study): 2011/1947 AM

ID. campione (ID. sample): 112926-S

Data inizio (Started on): 30/11/11

Microorganismi test (Test Microorganisms)	Dil	N		Nv		A		B		C	
		ufc/piastra (cfu/plate)	ufc/piastra (cfu/plate)	ufc/piastra (cfu/plate)	ufc/piastra (cfu/plate)	ufc/piastra (cfu/plate)	ufc/piastra (cfu/plate)	ufc/piastra (cfu/plate)	ufc/piastra (cfu/plate)	ufc/piastra (cfu/plate)	ufc/piastra (cfu/plate)
Staphylococcus aureus ATCC6538	-6	>330	>330	68	69	74	71	82	69	73	80
	-7	21	39								
		8.48	VALIDO (VALID)	6.9E+02		7.3E+01		7.6E+01		7.7E+01	
Pseudomonas aeruginosa ATCC15442	-6	>330	>330	82	73	59	63	67	80	62	50
	-7	42	40								
		8.61	VALIDO (VALID)	7.8E+02		6.1E+01		7.4E+01		5.6E+01	

N: conteggio sospensione batterica ufc/ml (N: count of the bacterial suspension cfu/ml)

Nv: conteggio sospensione batterica per il saggio preliminare ufc/ml (Nv: count of the bacterial suspension in the preliminary assay cfu/ml)

A: conteggio nella convalida delle condizioni sperimentali ufc/ml (A: count in the experimental conditions verification solution cfu/ml)

B: conteggio nel controllo di tossicità del neutralizzante ufc/ml (B: count in the neutraliser toxicity control cfu/ml)

C: conteggio nel controllo dell'efficacia del neutralizzante ufc/ml (C: count in the neutraliser effectiveness control cfu/ml)

eurofins	Prova quantitativa in sospensione per la valutazione dell'attività battericida di base dei disinfettanti chimici e antisettici (Quantitative suspension test for the evaluation of basic bactericidal activity of chemical disinfectants and antiseptics)
Mod. PS/MIC/001.E	Norma (Standard): EN 1040:2005- phase 1/step 1
Rev.3	Pagina 2 di 2 (page 2 of 2)

ID. studio (ID. Study): 2011/1947 AM ID. campione (ID. sample): 112926-S
 Data inizio (Started on): 30/11/11

Microorganismi test (Test Microorganisms)	CONCENTRAZIONI E TEMPI DI CONTATTO ufc/piastra (CONCENTRATIONS AND CONTACT TIMES cfu/plate)					
	80%	5 MIN	25%	5 MIN	10%	5 MIN
Staphylococcus aureus ATCC6538	0	0	0	0	21	21
	Na= <	2.15	Na= <	2.15	Na=	2.32
	R= >	5.33	R= >	5.33	R=	5.16
Pseudomonas aeruginosa ATCC15442	0	0	>330	>330	>330	>330
	Na= <	2.15	Na= >	3.52	Na= >	3.52
	R= >	5.46	R= <	4.09	R= <	4.09

Na = conteggio della miscela test ufc/ml (Na = count of the test mixture cfu/ml)
 R = riduzione della vitalità (R = vitality reduction)

Sigla tecnico (Technician signature):  Data fine (Finished on): 02/12/11
 Sigla Approvazione (Approval signature):  Data (Date): 02/12/11