Final report 2011/2133 AMi

SUSPENSION BASIC FUNGICIDAL EFFECTIVENESS ON VIRES 5

Study Program No: 2011/2133 AM

Contract No: PARA2011040201

Sponsor: VIRES5 BVBA
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2990 WUUSTWEZEL (BELGIUM)

Study monitor: BSL BIOSERVICE SCIENTIFIC LABORATORIES GmbH
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Test substance: VIRES 5

Director of the Study: .....
(Laura Brambilla)

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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 affected the quality or integrity of the study

Study Director
(Laura Brambilla)

Jan 09th 2012
DATE

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	December, 20th 2011
- Amendment #1 to the Study program	December, 22nd 2011
- Raw data	January, 9th 2012
- Final report	January, 9th 2012

Patrizia Custode
QA MANAGER
(Patrizia Custode)

Jan. 09th, 2012
DATE

SUMMARY

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

The suspension fungicidal effectiveness was evaluated with the following experimentation:
- phase 1, basic fungicidal activity suspension test for chemical disinfectants and antiseptics in which 2 different fungal strains, *Candida albicans* ATCC 10231 and *Aspergillus niger* ATCC 16404 were exposed to the test substance in the following conditions:
- final concentrations: 80% (maximum concentration testable) – 50% - 25%
- contact times: 5 – 15 minutes
- temperature: 20 ± 1°C

On the basis of the results obtained in compliance with the assay validity criteria, the test substance VIRES 5 results **FUNGICIDAL** with the concentration of 80% after 15 minutes of contact time, in compliance with EN 1275:2005.

See Experimental Report 2011/2133 for more details.

INTRODUCTION

A study was conducted on behalf of VIRES5 BVBA in order to demonstrate the basic fungicidal 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. Buozzi n. 2 (Italy).

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).

EXPERIMENTATION	*START*	*END*	*RESEARCHER*
Basic fungicidal activity suspension test	Dec 28th 2011	Jan 02nd 2012	C. Meroni

On December 22nd, 2011 an amendment to the Study Program 2011/2133 AMi was issued in order to add some information about the analyzed sample provided by the Sponsor and to correct the foreseen study ending date for a clerical error.

TERMS AND DEFINITIONS

Fungicidal: a chemical agent or formulation that kills fungi and their spores under given conditions.

Fungicidal activity: the capability of a product of reducing the number of vegetative yeasts and mycotic spores under given conditions.

REFERENCES

EN 1275, December 2005 - Chemical disinfectants and antiseptics – Quantitative suspension test for the evaluation of basic fungicidal or basic yeasticidal 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.
The retained sample will be kept until July 2014 according to the expiry date provided by the Sponsor.
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.

TEST SUBSTANCE

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

Name	VIRES 5
Product	Purified water with increased OrpV value
Stability	3 years
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	July 2011
Expiry date	July 2014
CoA	Not provided
Receiving n.	EUITVI-21918
Receiving date	Dec 14th 2011
Id number	11.3172-S

The characterisation of the test substance is responsibility of the Customer.

Experimental Report 2011/2133 - EVALUATION OF BASIC FUNGICIDAL ACTIVITY IN SUSPENSION. DILUTION – NEUTRALIZATION METHOD (EN1275:2005)

EXPERIMENTAL PROCEDURE

1 ASSAY SYSTEM

Microorganisms
The following test strains were used:

Candida albicans	ATCC 10231
Aspergillus niger	ATCC 16404

Conservation
Candida albicans strain was kept frozen; before using it was transplanted on Malt Extract Agar and kept in a refrigerator at 5°C ±3°C.
Aspergillus niger strain was kept in a refrigerator at 5°C ±3°C on Malt Extract Agar.

Preparation of the yeast suspension
Candida albicans strain was transplanted on Malt Extract Agar slant twice consecutively and incubated at 30°C ±1°C for 42 to 48 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 count of about $1.5x10^7$ to $5.0x10^7$ cfu/ml.
Dilutions to 10^{-5} and 10^{-6} were prepared in an diluent to perform the count of the fungine suspensions.
A double count through inclusion in Agar was performed. The plates were incubated at 30°C ±1°C for 48 hours. The number of cfu/ml was determined at the end of the incubation period. **N** value was then calculated.

Preparation of the fungal spore suspensions
Two plates containing Malt Extract Agar were inoculated proceeding from the conservation slants using glass beads by adding 5 ml of a sterile solution 0.05% polysorbate 80. The plates were incubated at 30°C ± 1°C for 5-7 days. Then, by adding 10 ml of a sterile solution 0.05% polysorbate 80, a conidiospore suspension was obtained. After careful stirring, the suspension was transferred into a sterile test tube and filtered to remove any existing mycelia.
The spores were diluted to a concentration of about $1.5x10^7$ to $5.0x10^7$ cfu/ml. Dilutions to 10^{-5} and 10^{-6} were prepared in an eluant to perform the count of the fungine suspensions. A double count through inclusion in Agar was performed.
The plates were incubated at 30°C ± 1°C for 42 to 48 hours. After the incubation period the uncountable plates were eliminated and the remaining plates were incubated for additional 48 hours and, if necessary for 20-24 hours more. The number of cfu/ml was determined at the end of the incubation period. **N** value was then calculated.

2 CULTURAL MEDIA AND REAGENTS

Malt extract Agar (MEA) MERCK

Diluent

Casein tryptone	1.0g	MERCK
NaCl	8.5g	MERCK
Distilled water q. s. to	1000 ml	

Water (WFI) EUROSPITAL

3 EQUIPMENT

Dry sterilization oven	MEMMERT
Steam autoclave	FEDEGARI
Incubator	GALLI
pHmeter	BECKMAN
Vortex stirrer	VELP
Chronometer	ARBORE
Micropipettes	GILSON
Spectrophotmeter	SHIMADZU

4. EXPERIMENTAL DESIGN

Test temperature
The test was performed at 20°C ±1°C.

Experimental conditions
The test was performed at the following conditions:
- final concentrations: 80% (maximum concentration testable) – 50% – 25%
- contact times: 5 - 15 minutes

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

Neutraliser
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
Triptone-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 assay sample, the fungine suspensions and the diluent had previously been stabilized at the test temperature, while the neutraliser and the water had been stabilized at 20°C ±1°C.

Count of the fungine suspensions
The fungine suspensions were diluted to a concentration of $3.0x10^2$ to $1.6x10^3$ cfu/ml.
This suspension was further diluted using a decimal dilution and the suspension number of colony-forming units was then determined through inclusion in agar. After that the plates containing *Candida albicans* were incubated at 30°C ±1°C for 48 hours.
The plates containing *Aspergillus niger* were incubated at 30°C ±1°C for 42 to 48 hours; after the incubation period the uncountable plates were eliminated and the remaining plates were incubated for additional 48 hours and, if necessary for 20-24 hours more.
N_v value was then calculated.

Preparation of test substance solution
The test substance was diluted up to the highest concentration tested in the assay.

Validation of the experimental conditions
1 ml of water and 1 ml of fungine suspension containing from $3.0x10^2$ to $1.6x10^3$ cfu/ml were placed in a test tube.
The components were left in contact for 2 minutes at the test temperature, afterwards 8 ml of water were added and left in contact at the temperature adopted during the assay for the longest period to be tasted.
At the end of the contact time, the mixture was vortexed and a double count was performed by inclusion in agar.
The plates containing *Candida albicans* were incubated at 30°C ±1°C for 48 hours.
The plates containing *Aspergillus niger* were incubated at 30°C ±1°C for 42 to 48 hours. After the incubation period the uncountable plates were eliminated and the remaining plates were incubated for additional 48 hours and, if necessary for 20-24 hours more.
After this period the number of colony forming units per ml of the mixture was determined and **A** value was calculated.

Verification of the neutraliser non-toxicity
For each test strain, 8 ml of neutraliser, 1 ml of distilled water and 1 ml of fungine suspension containing from $3.0x10^2$ to $1.6x10^3$ cfu/ml were mixed in a test tube and left at 20°C ±1°C for 5 minutes.
At the end of the contact time, the mixture was vortexed and a double count was performed by inclusion in agar.
The plates containing *Candida albicans* were incubated at 30°C ±1°C for 48 hours.
The plates containing *Aspergillus niger* were incubated at 30°C ±1°C for 42 to 48 hours. After the incubation period the uncountable plates were eliminated and the remaining plates were incubated for additional 48 hours and, if necessary for 20-24 hours more.
After this period the number of colony forming units per ml was determined and **B** value was then calculated.

Validation of the dilution-neutralization test
For each test strain, 1 ml of water, 1 ml of diluent and 8 ml of test substance at the highest concentration used in the assay were mixed in a test tube and left in contact at 20°C ±1°C for the longest period to be tested. At the end of the contact time, 1 ml mixture was transferred into a test tube containing 8 ml neutraliser and left in contact at 20°C ±1°C for 5 minutes. Afterwards 1 ml of the fungine suspension (with a concentration range of $3.0x10^2$-$1.6x10^3$ cfu/ml) was added and this preparation was left in contact at 20°C ±1°C for 30 minutes.
At the end of the contact time, the mixture was vortexed and a double count was performed by inclusion in agar.
The plates containing *Candida albicans* were incubated at 30°C ±1°C for 48 hours.
The plates containing *Aspergillus niger* were incubated at 30°C ±1°C for 42 to 48 hours. After the incubation period the uncountable plates were eliminated and the remaining plates were incubated for additional 48 hours and, if necessary for 20-24 hours more.
After this period the number of colony forming units per ml was determined and **C** value was then calculated.

5.2 *Assay*

The assay sample, the fungine suspensions and the diluent had previously been stabilized at the test temperature, while the neutraliser and the water had been stabilized at 20°C ±1°C.
For each fungine strain and each test substance concentration, a test tube containing 1 ml of water and 1 ml of fungine suspension showing concentration in a 1.5 $x10^7$ to 5.0$x10^7$ cfu range, was prepared at the temperature adopted during the assay.
After a contact time of 2 minutes, 8 ml of test substance have been added and left in contact at the test temperature for the set time.
At the end of the contact time (15 minutes), 1 ml of the mixture was transferred into a test tube containing 8 ml neutraliser and 1 ml of distilled water. After 5 minutes of neutralisation at 20°C ±1°C the mixture was then vortexed and a double count was performed by inclusion in agar.
The plates containing *Candida albicans* were incubated at 30°C ±1°C for 48 hours.
The plates containing *Aspergillus niger* were incubated at 30°C ±1°C for 42 to 48 hours. After the incubation period the uncountable plates were eliminated and the remaining plates were incubated for additional 48 hours and, if necessary for 20-24 hours more.
After this period the number of colony forming units per ml was determined and **Na** value was then calculated.

6 CALCULATION AND EXPRESSION OF THE RESULTS

Calculation of the fungine count (cfu/ml)
The counting was performed using the number of colonies counted on both plates.
Only the plates showing a number of colonies included in a 15-150 range for moulds and in a 15-300 range for yeasts were used to perform the result calculation. A deviation of 10% is accepted, so the limits are 14 and 165 for moulds and 14 and 330 for yeasts.
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$x10^2$.
Where the number of cfu on every plate counted is >165 (or >330), the number of cfu/ml should be recorded as >1.65 x 10^3 (or >3.3$x10^3$).

Test suspension
The calculation of the microbial counting for the suspension test (**N**) is performed applying the following formula:

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

where:
c = sum of colonies counted on both plates
n_1 = number of counted plates in the lower dilution
n_2 = number of counted plates in the highter dilution
d = dilution factor corresponding to the lower dilution

Assay and preliminary assay
For the calculation of the bacterial counting for the assay (**Na**) and for the preliminary assay (**A**, **B**, **C** and $\mathbf{N_V}$) 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
N_0 = N/10
Na = microbial counting for the test mixture at the end of the contact time

ASSAY VALIDITY CRITERIA

Verify the following:

N: must be included between $1.5x10^7$ and $5.0x10^7$ cfu/ml

N_V: must be included between $3.0x10^2$ and $1.6x10^3$ cfu/ml

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

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

where:

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

N_V: count of cfu/ml in the fungine 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 fungicidal when the fungine count for each strain is reduced by at least 4 Log following 15 minutes' contact at 20°C.

The test substance is considered effective against the test microorganisms when the fungine count for each strain is reduced by at least 4 Log following the chosen contact time at 20°C.

RESULTS

Preliminary assay
N, Nv, A, B, C and control of weighted mean counts values found for each fungal strain comply with assay validity criteria. The values are reported separately in Attachment #1.

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

TEST MICROORGANISMS	CONTACT TIME AND TESTED CONCENTRATIONS		
	80%	**50%**	**25%**
	5 minutes		
Candida albicans ATCC10231	3.52	<3.05	<3.05
Aspergillus niger ATCC16404	<3.18	<3.18	<3.18
	15 minutes		
Candida albicans ATCC10231	>4.42	3.89	<3.05
Aspergillus niger ATCC16404	>4.25	3.77	<3.18

DEVIATIONS

No deviations to the study program occurred.

CONCLUSIONS

On the basis of the results obtained in compliance with the assay validity criteria, the test substance VIRES 5 results **FUNGICIDAL** with the concentration of 80% after 15 minutes of contact time, in compliance with EN 1275:2005.

ATTACHMENTS

ATTACHMENT	TITLE	NUMBER OF PAGES
N.1	EXCEL ELABORATION OF EXPERIMENTATION 2011/2133	3

eurofins | biolab	**Prova quantitativain sospensione per la valutazione dell'attività fungicida e lieviticida di base di disinfettanti chimici e antisettici** ***(Quantitative suspension test for the evaluation of basic fungicidal or basic yeasticidal activity of chemical disinfectants and antiseptics)***
Mod. PS/MIC/019.E	**Norma *(Standard)*** : EN 1275:2005 - phase 1
Rev.3	Pagina 1 di 3 *(page 1 of 3)*

Data inizio *(Started on)*: 28/12/11

ID. studio *(ID. Study)*: 2011/2133 AM

ID. campione *(ID. sample)*: 11.3172-S

Microrganismi test *(Test Microrganisms)*		N		Nv		A		B		C	
	Dil	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)*
Candida albicans ATCC10231	-5	>330	>330	66	69	52	53	44	60	53	57
	-6	35	39								
		7.57	VALIDO (VALID)	6.8E+02		5.3E+01		5.2E+01		5.5E+01	
Aspergillus niger ATCC16404	-5	>165	>165	70	50	40	30	40	40	60	50
	-6	28	22								
		7.40	VALIDO (VALID)	6.0E+02		3.5E+01		4.0E+01		5.5E+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*)

Data inizio *(Started on)*: 28/12/11

ID. studio *(ID. Study)*: 2011/2133 AM

ID. campione *(ID. sample)*: 11.3172-S

Microrganismi test *(Test Microrganisms)*		CONCENTRAZIONI E TEMPI DI CONTATTO ufc/piastra *(CONCENTRATIONS AND CONTACT TIMES cfu/plate)*					
		80%	5 min	50%	5 min	25%	5 min
Candida albicans ATCC10231		102	122	>330	>330	>330	>330
		Na=	3.05	Na= >	3.52	Na= >	3.52
		R=	3.52	R= <	3.05	R= <	3.05
Aspergillus niger ATCC16404		>165	>165	>165	>165	>165	>165
		Na= >	3.22	Na= >	3.22	Na= >	3.22
		R= <	3.18	R= <	3.18	R= <	3.18

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)*:

Sigla Approvazione *(Approval signature)*:

Data fine *(Finished on)*: 02/01/12

Data *(Date)*: 02/01/12

Data inizio *(Started on)*: 28/12/11

ID. studio *(ID. Study)*: 2011/2133 AM

ID. campione *(ID. sample)*: 11.3172-S

Microrganismi test *(Test Microrganisms)*		CONCENTRAZIONI E TEMPI DI CONTATTO ufc/piastra *(CONCENTRATIONS AND CONTACT TIMES cfu/plate)*					
		80%	15 min	50%	15 min	25%	15 min
Candida albicans ATCC10231		0	0	50	46	>330	>330
		Na= < 2.15		Na= 2.68		Na= > 3.52	
		R= > 4.42		R= 3.89		R= < 3.05	
Aspergillus niger ATCC16404		1	0	47	38	>165	>165
		Na= < 2.15		Na= 2.63		Na= > 3.22	
		R= > 4.25		R= 3.77		R= < 3.18	

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)*:

Sigla Approvazione *(Approval signature)*:

Data fine *(Finished on)*: 02/01/12

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