| | CENTRO DI SAGGIO BPL<br>Certificazione n. 154/217/2002 | IMS srl<br>Via Laurentina 169, Pomezia (RM)<br>Tel. 06 9145399 Fax 06 9146099 |
|---|---|---|
| Programma di studio 37 | Rapporto finale 01 | Copia finale |
| Direttore dello studio: Dott. Giancarlo Folchitto | | Data: 20.07.2004 |


# Microbiological Study

## Product Code number : VRKS

**Sponsor: UNITEC – L.go Zandonai, 3 – Milano (Italy)**

**The purpose of this study is to investigate the microbiological activity of the product in according to the procedure UNI EN 1656: "Quantitative suspension test for the evaluation of bactericidal activity of chemical disinfectants and antiseptics used in veterinary field. Test method and requirements (phase 2/step 1)"**

Study Director

Giancarlo Folchitto

# CONTENTS

## INTRODUCTION

From UNITEC (Milan-Italy) we received samples of product to determine the microbiological characteristics, in the label claimed.

COMPOSITION: Potassium peroxomonosulphate, anionic surfactant, organic acids and inorganic salts.

INDICATIONS. Veterinary and animal husbandry disinfectant. May be used in food processing plants and hatcheries.

DILUTIONS: 1:100 (bacteria & viruses)

1:33 (fungi)*

* on pre-cleaned surfaces

We have tested the dilution 1:100, 1:80 and 1:250.

The dilution 1:250 has been tested as non-active dilution, in accordance with the method.

Contact time: 30 e 60 min.

## FOREWORD

On the label is printed:

batch number: BN 17176

Manufactured: 28.04.03

Exp. 28.10.04.

Identification of the product: the product has been identified with the code number

**VRKS**

Date of receipt: 09.06.04

## SCOPE OF THE TEST

The product, diluted in hard water when tested, in accordance with the procedure, shall demonstrate at least a $10^5$ reduction in viable counts, when the test organisms are *Enterococcus hirae, Proteus vulgaris, Pseudomonas aeruginosa, Staphylococcus aureus.*

The test is carried out under simulated low or high soiling conditions, according to its practical applications and under the required test conditions.

## REQUIREMENTS

The procedure specifies a test method (phase 2/step 1) and the minimum requirements for bactericidal activity of chemical disinfectant and antiseptic products that form a homogeneous physically stable preparation in standardized hard water.

The European Standard is applicable to products for use in veterinary field i.e. in the breeding, husbandry, production, transport and disposal of all animals except when in food chain following death and entry to the processing industry.

| Information refers to the original batch | |
|---|---|
| Code number | VRKS |
| Batch number | BN 17176 |
| Expiration date | 28.10.2004 |
| Manufactured by | Antec International Limited |
| Storage conditions | room temperature |
| Received by Laboratory | 09.06.2004 |
| Start of analisys | 10.06.2004 |
| End of analisys | 28.06.2004 |
| **GENERAL** | |
| Diluent of the sample | Standardized hard water |
| Product test solution | 1 to 100, 1 to 80, 1 to 250 in standardized hard water |
| Interfering substances | low level soiling: BSA 3 g/lt in sterile distilled water<br>high level soiling: yest extract powder 10 g/lt + BSA 10 g/lt in sterile distilled water |
| Contact time | 30 & 60 min |
| Contact temperature | 10°± 1°C e 30°± 1°C |
| Neutralizer | Membrane filtration |
| Incubation temperature | 36°C ± 1°C |

**Materials and reagents**

- Product test solution: prepared in standadized hard water at three different concentrations: two concentrations in the active range, one concentrations in the non active range.
- Test organisms:

| | |
|---|---|
| *Enterococcus hirae* | ATCC 10541 |
| *Proteus vulgaris* | ATCC 13315 |
| *Pseudomonas aeruginosa* | ATCC 15442 |
| *Staphylococcus aureus* | ATCC 6538 |

- Tryptone Soya Agar (TSA – for maintenance of bacterial strains and performance of viable counts)

| | | |
|---|---|---|
| Tryptone, pancreatic digest of casein | g | 15,0 |
| Soya peptone, papaic digest of Soybean meal | g | 5,0 |
| NaCl | g | 5,0 |
| Agar | g | 15,0 |
| Distilled water to | ml | 1.000,0 |

Sterilize in the autoclave – pH (after sterilization) = 7,2 ± 0,2 (a 20°C).

- Diluent (TSC):

| | | |
|---|---|---|
| Triptone, pancreatic digest of casein | g | 1,0 |
| NaCl | g | 8.5 |
| Distilled water to | ml | 1.000,0 |

Sterilizze in the autoclave – pH (after sterilization) = 7,0 ± 0,2 (a 20°C).

- Neutralizer: membrane filtration.
- Rinsing liquid (for membrane filtration): sterile distilled water.
- Standardized hard water for dilution of products, prepared as follows:

Soluzion A: dissolve 19,84 g of anhydrous $MgCl_2$ and 46,24 g anhydrous $CaCl_2$ in sterile distilled water and make up to 1000 ml. Sterilize in the autoclave 121°C ± 3°Cfor a minimum of holding time of 5 min.

Solution B: dissolve 35,02 g of $NaHCO_3$ in sterile distilled water and dilute to 1000 ml.

Sterilize by passing trough a filter with a maximum effective pore size of 0,22 µm.

Add at least 600 ml of sterile distilled water to 6 ml of solution A in a sterile 1000 ml volumetric flask, then add 8 ml of solution B and make up to 1000 ml with sterile distilled

water.

The pH of the solution shall be 7,0 ± 0,2 before use.

The water shall be freshly prepared, i.e. not used for more than one day.

- Interfering substances

1) bovine albumin solution for low level soiling conditions.

Dissolve 3 g of bovine albumin in 90 ml of sterile distilled water in a 100 ml volumetric flask. Make up to the mark with sterile distilled water. Sterilize by membrane filtration.

The final concentration of bovine albumin in the test procedure is 3 g/l.

2) Albumin/yeast extract mixture for high level soiling.

a) Dissolve 50 g yeast extract powder in 150 ml of sterile distilled water in a 250 ml volumetric flask and allow foam to collapse. Make up to the mark with water. Transfer to a clean dry bottle and sterilize in the autoclave. Allow to cool to 20°C ± 5°C.

b) Pipette 25 ml of this solution into a 50 ml volumetric flask and add 10 ml of sterile distilled water. Dissolve 5 g of the bovine albumin in the solution in the flask with shaking and allow foam to collapse. Make up to the mark with sterile distilled water and sterilize by filtration and keep in 10 ml portions in a refrigerator (at 2°C to 8°C) until use.

The final concentration in the test procedure is 10 g/l yeast extract and 10 g/l bovine albumin.

**- Apparatus and glassware**

- Autoclave
- Water baths
- Incubator
- pH-meter
- Spectrophotometer
- Stopwatch
- Vortex mixer
- Graduated pipettes
- Glass beads
- Petri dishes of size 90 mm to 100 mm
- Glass beads
- Volumetric flasks
- Mechanical shaker
- Membrane filtration apparatus
- Refrigerator
- Flow laminar cabinet

**Experimental conditions**

- Bacterial test suspension: 1,5 to 5 x $10^8$ cfu/ml ("N")

  For counting, prepare $10^{-6}$ and $10^{-7}$ dilutions of the test suspensions using diluent.

  Pipette each 1,0 ml sample in separate Petri dishes and add 12 to 15 ml melted TSA, cooled to 45°C ± 1°C.

  Incubate the plates at 36°C ± 1°C for 24 h.

  Count the plates and determine the number of colony forming units.

  * $\frac{150 + 164 + 14 + 19}{(2 + 0{,}1x2) \times 10^{-6}} = 1{,}58 \times 10^8$

| Test organism | cfu/ml | N |
|---|---|---|
| *Enterococcus hirae* | $10^{-6}$ 255; 245<br>$10^{-7}$ 25; 25 | 2,5 x $10^8$ |
| *Proteus vulgaris* | $10^{-6}$ 250; 230<br>$10^{-7}$ 25; 24 | 2,4 x $10^8$ |
| *Pseudomonas aeruginosa* | $10^{-6}$ 230; 212<br>$10^{-7}$ 23; 21 | 2,2 x $10^8$ |
| *Staphylococcus aureus* | $10^{-6}$ 224; 231<br>$10^{-7}$ 23; 23 | 2,3 x $10^8$ |

- Product test solution:

  VRKS diluted in standardized hard water 1:80 – 1:100 – 1:250

- Temperature to be tested: 10°C ± 1°C and 30°C ± 1°C

- Contact time: 30 & 60 min ± 10 s

**Test procedure – Membrane filtration method**

Prior to testing, equilibrate all reagents (product test solutions, bacterial test suspension interfering substances) to the test temperature.

Check that the temperature of the reagents is stabilized at the test temperature of 10°C ± 1°C and 30°C ± 1°C.

Pipette 1,0 ml of the interfering substance into a test tube.

Add 1,0 ml of one of the bacterial test suspensions.

Start the stopwatch, immediately, mix and place the test tube in the water bath at 10°C ± 1°C (alternatively at 30°C ± 1°C) for 2 min ± 10 s. At the end of this time, add 8,0 ml of one of the product test solutions. Restart the stopwatch, mix and place the test tube in the water bath controlled at 10°C ± 1°C and 30°C ± 1°C for 30 and 60 min ± 10 s.

Just before the end of contact time, mix.

At the end of contact time pipette two samples of 0,1 ml of the test mixture and transfer each sample into a separate membrane filtration apparatus equipped with a membrane and containing 50 ml of the rinsing liquid (equilibrated at a temperature of 20°C ± 1°C).

Filter immediately.

Rinse with at least 150 ml but not more 500 ml of rinsing liquid, then transfer the membranes to the surface of two separate TSA plates.

This procedure is performed using the other product test solutions and the other bacterial test suspensions.

**Counting of test mixture**

Incubate the plates at 36°C ± 1°C per 24 h.

Calculate the number of cfu/ml in the test mixture $N_a$.

Where the number of cfu is lower than 15, the viable count is recorded as less than $1,5 \times 10^2$ cfu/ml (< $1,5 \times 10^2$ cfu/ml).

Where the number of cfu is higher than 300 the viable count is recorded as higher than $3 \times 10^3$ cfu/ml (> $3 \times 10^3$ cfu/ml).

**Test results for three concentrations of VRKS, plate counts**

**Contact time: 30 min ± 10 s**

**Temperature: 10°C ± 1°C**

| Test organism | Conditions: low level soiling | | | |
|---|---|---|---|---|
| | N | 1:250 | 1:80 | 1:100 |
| *Enterococcus hirae* ATCC 10541 | $2,5 \times 10^8$ | > 300; > 300 | 16; 14 | 21; 23 |
| *Proteus vulgaris* ATCC 13315 | $2,4 \times 10^8$ | > 300; > 300 | 12; 9 | 15; 14 |
| *Pseudomonas aeruginosa* ATCC 15442 | $2,2 \times 10^8$ | > 300; > 300 | 12; 15 | 14; 14 |
| *Staphylococcus aureus* ATCC 6538 | $2,3 \times 10^8$ | > 300; > 300 | 13; 16 | 16; 15 |

| Test organism | Conditions: high level soiling | | | |
|---|---|---|---|---|
| | N | 1:250 | 1:80 | 1:100 |
| *Enterococcus hirae* ATCC 10541 | $2,5 \times 10^8$ | > 300; > 300 | 23; 27 | 30; 29 |
| *Proteus vulgaris* ATCC 13315 | $2,4 \times 10^8$ | > 300; > 300 | 18; 19 | 22; 20 |
| *Pseudomonas aeruginosa* ATCC 15442 | $2,2 \times 10^8$ | > 300; > 300 | 16; 15 | 20; 21 |
| *Staphylococcus aureus* ATCC 6538 | $2,3 \times 10^8$ | > 300; > 300 | 19; 23 | 21; 22 |

Na: number of cfu/ml in the test mixture

Dilution factor = $10^{-1}$

**Test results for three concentrations of VRKS, plate counts**

**Contact time: 60 min ± 10 s**

**Temperature: 10°C ± 1°C**

| Test organism | Conditions: low level soiling | | | |
|---|---|---|---|---|
| | N | 1:250 | 1:80 | 1:100 |
| *Enterococcus hirae* ATCC 10541 | $2,5 \times 10^8$ | > 300; > 300 | 8; 7 | 10; 13 |
| *Proteus vulgaris* ATCC 13315 | $2,4 \times 10^8$ | > 300; > 300 | 4; 5 | 9; 6 |
| *Pseudomonas aeruginosa* ATCC 15442 | $2,2 \times 10^8$ | > 300; > 300 | 5; 6 | 7; 7 |
| *Staphylococcus aureus* ATCC 6538 | $2,3 \times 10^8$ | > 300; > 300 | 7; 5 | 13; 10 |

| Test organism | Conditions: high level soiling | | | |
|---|---|---|---|---|
| | N | 1:250 | 1:80 | 1:100 |
| *Enterococcus hirae* ATCC 10541 | $2,5 \times 10^8$ | > 300; > 300 | 13; 10 | 15; 12 |
| *Proteus vulgaris* ATCC 13315 | $2,4 \times 10^8$ | > 300; > 300 | 10; 10 | 15; 13 |
| *Pseudomonas aeruginosa* ATCC 15442 | $2,2 \times 10^8$ | > 300; > 300 | 10; 9 | 14; 13 |
| *Staphylococcus aureus* ATCC 6538 | $2,3 \times 10^8$ | > 300; > 300 | 6; 7 | 7; 8 |

Na: number of cfu/ml in the test mixture

Dilution factor = $10^{-1}$

**Contact time: 30 min ± 10 s**

**Temperature: 30°C ± 1°C**

| Test organism | N | Conditions: low level soiling | | |
|---|---|---|---|---|
| | | 1:250 | 1:80 | 1:100 |
| *Enterococcus hirae* ATCC 10541 | $2,5 \times 10^8$ | > 300; > 300 | 12; 12 | 14; 18 |
| *Proteus vulgaris* ATCC 13315 | $2,4 \times 10^8$ | > 300; > 300 | 9; 9 | 10; 11 |
| *Pseudomonas aeruginosa* ATCC 15442 | $2,2 \times 10^8$ | > 300; > 300 | 6; 5 | 10; 10 |
| *Staphylococcus aureus* ATCC 6538 | $2,3 \times 10^8$ | > 300; > 300 | 9; 8 | 13; 15 |

| Test organism | N | Conditions: high level soiling | | |
|---|---|---|---|---|
| | | 1:250 | 1:80 | 1:100 |
| *Enterococcus hirae* ATCC 10541 | $2,5 \times 10^8$ | > 300; > 300 | 16; 20 | 18; 21 |
| *Proteus vulgaris* ATCC 13315 | $2,4 \times 10^8$ | > 300; > 300 | 15; 15 | 17; 16 |
| *Pseudomonas aeruginosa* ATCC 15442 | $2,2 \times 10^8$ | > 300; > 300 | 9; 11 | 12; 13 |
| *Staphylococcus aureus* ATCC 6538 | $2,3 \times 10^8$ | > 300; > 300 | 14; 17 | 16; 16 |

**Contact time: 60 min ± 10 s**

**Temperature: 30°C ± 1°C**

| Test organism | N | Conditions: low level soiling | | |
|---|---|---|---|---|
| | | 1:250 | 1:80 | 1:100 |
| *Enterococcus hirae* ATCC 10541 | $2,5 \times 10^8$ | > 300; > 300 | 9; 10 | 10; 12 |
| *Proteus vulgaris* ATCC 13315 | $2,4 \times 10^8$ | > 300; > 300 | 7; 7 | 10; 7 |
| *Pseudomonas aeruginosa* ATCC 15442 | $2,2 \times 10^8$ | > 300; > 300 | 2; 3 | 4; 5 |
| *Staphylococcus aureus* ATCC 6538 | $2,3 \times 10^8$ | > 300; > 300 | 5; 5 | 6; 7 |

| Test organism | N | Conditions: high level soiling | | |
|---|---|---|---|---|
| | | 1:250 | 1:80 | 1:100 |
| *Enterococcus hirae* ATCC 10541 | $2,5 \times 10^8$ | > 300; > 300 | 11; 13 | 14; 14 |
| *Proteus vulgaris* ATCC 13315 | $2,4 \times 10^8$ | > 300; > 300 | 9; 10 | 10; 10 |
| *Pseudomonas aeruginosa* ATCC 15442 | $2,2 \times 10^8$ | > 300; > 300 | 4; 4 | 4; 5 |
| *Staphylococcus aureus* ATCC 6538 | $2,3 \times 10^8$ | > 300; > 300 | 5; 6 | 7; 7 |

**Reduction in viability (R) of VRKS at test concentration and temperature indicated**

R = reduction viability = $\frac{N \times 10^{-1}}{Na \text{ (colony number x 10)}}$

**Contact time: 30 min ± 10 s**

**Temperature: 10°C ± 1°C**

| Test organism | N | Conditions: low level soiling | | |
|---|---|---|---|---|
| | | 1:250 | 1:80 | 1:100 |
| *Enterococcus hirae* ATCC 10541 | $2,5 \times 10^8$ | R < 5 log | R > 5 ($1,5 \times 10^5$) | R > 5 ($1,14 \times 10^5$) |
| *Proteus vulgaris* ATCC 13315 | $2,4 \times 10^8$ | R < 5 log | R > 5 ($2,10 \times 10^5$) | R > 5 ($1,71 \times 10^5$) |
| *Pseudomonas aeruginosa* ATCC 15442 | $2,2 \times 10^8$ | R < 5 log | R > 5 ($1,69 \times 10^5$) | R > 5 ($1,57 \times 10^5$) |
| *Staphylococcus aureus* ATCC 6538 | $2,3 \times 10^8$ | R < 5 log | R > 5 ($1,64 \times 10^5$) | R > 5 ($1,44 \times 10^5$) |

| Test organism | N | Conditions: high level soiling | | |
|---|---|---|---|---|
| | | 1:250 | 1:80 | 1:100 |
| *Enterococcus hirae* ATCC 10541 | $2,5 \times 10^8$ | R < 5 log | R > 5 ($1,0 \times 10^5$) | $8,60 \times 10^4$ |
| *Proteus vulgaris* ATCC 13315 | $2,4 \times 10^8$ | R < 5 log | R > 5 ($1,3 \times 10^5$) | R > 5 ($1,14 \times 10^5$) |
| *Pseudomonas aeruginosa* ATCC 15442 | $2,2 \times 10^8$ | R < 5 log | R > 5 ($1,43 \times 10^5$) | R > 5 ($1,1 \times 10^5$) |
| *Staphylococcus aureus* ATCC 6538 | $2,3 \times 10^8$ | R < 5 log | R > 5 ($1,15 \times 10^5$) | R > 5 ($1,07 \times 10^5$) |

**Contact time: 60 min ± 10 s**

**Temperature: 10°C ± 1°C**

| Test organism | N | Conditions: low level soiling | | |
|---|---|---|---|---|
| | | 1:250 | 1:80 | 1:100 |
| *Enterococcus hirae* ATCC 10541 | $2,5 \times 10^8$ | R < 5 log | R > 5 ($3,6 \times 10^5$) | R > 5 ($2,5 \times 10^5$) |
| *Proteus vulgaris* ATCC 13315 | $2,4 \times 10^8$ | R < 5 log | R > 5 ($6,0 \times 10^5$) | R > 5 ($3,43 \times 10^5$) |
| *Pseudomonas aeruginosa* ATCC 15442 | $2,2 \times 10^8$ | R < 5 log | R > 5 ($3,7 \times 10^5$) | R > 5 ($3,17 \times 10^5$) |
| *Staphylococcus aureus* ATCC 6538 | $2,3 \times 10^8$ | R < 5 log | R > 5 ($3,29 \times 10^5$) | R > 5 ($2,09 \times 10^5$) |

| Test organism | N | Conditions: high level soiling | | |
|---|---|---|---|---|
| | | 1:250 | 1:80 | 1:100 |
| *Enterococcus hirae* ATCC 10541 | $2,5 \times 10^8$ | R < 5 log | R > 5 ($2,27 \times 10^5$) | R > 5 ($1,92 \times 10^5$) |
| *Proteus vulgaris* ATCC 13315 | $2,4 \times 10^8$ | R < 5 log | R > 5 ($2,4 \times 10^5$) | R > 5 ($1,71 \times 10^5$) |
| *Pseudomonas aeruginosa* ATCC 15442 | $2,2 \times 10^8$ | R < 5 log | R > 5 ($2,22 \times 10^5$) | R > 5 ($1,59 \times 10^5$) |
| *Staphylococcus aureus* ATCC 6538 | $2,3 \times 10^8$ | R < 5 log | R > 5 ($3,29 \times 10^5$) | R > 5 ($3,29 \times 10^5$) |

**Contact time: 30 min ± 10 s**

**Temperature: 30°C ± 1°C**

| Test organism | N | Conditions: low level soiling | | |
|---|---|---|---|---|
| | | 1:250 | 1:80 | 1:100 |
| *Enterococcus hirae* ATCC 10541 | $2,5 \times 10^8$ | R < 5 log | R > 5 ($2,08 \times 10^5$) | R > 5 ($1,56 \times 10^5$) |
| *Proteus vulgaris* ATCC 13315 | $2,4 \times 10^8$ | R < 5 log | R > 5 ($2,67 \times 10^5$) | R > 5 ($2,29 \times 10^5$) |
| *Pseudomonas aeruginosa* ATCC 15442 | $2,2 \times 10^8$ | R < 5 log | R > 5 ($4,04 \times 10^5$) | R > 5 ($2,22 \times 10^5$) |
| *Staphylococcus aureus* ATCC 6538 | $2,3 \times 10^8$ | R < 5 log | R > 5 ($2,71 \times 10^5$) | R > 5 ($1,64 \times 10^5$) |

| Test organism | N | Conditions: high level soiling | | |
|---|---|---|---|---|
| | | 1:250 | 1:80 | 1:100 |
| *Enterococcus hirae* ATCC 10541 | $2,5 \times 10^8$ | R < 5 log | R > 5 ($1,39 \times 10^5$) | R > 5 ($1,28 \times 10^5$) |
| *Proteus vulgaris* ATCC 13315 | $2,4 \times 10^8$ | R < 5 log | R > 5 ($1,60 \times 10^5$) | R > 5 ($1,45 \times 10^5$) |
| *Pseudomonas aeruginosa* ATCC 15442 | $2,2 \times 10^8$ | R < 5 log | R > 5 ($2,22 \times 10^5$) | R > 5 ($1,78 \times 10^5$) |
| *Staphylococcus aureus* ATCC 6538 | $2,3 \times 10^8$ | R < 5 log | R > 5 ($1,48 \times 10^5$) | R > 5 ($1,44 \times 10^5$) |

**Contact time: 60 min ± 10 s**

**Temperature: 30°C ± 1°C**

| Test organism | N | Conditions: low level soiling | | |
|---|---|---|---|---|
| | | 1:250 | 1:80 | 1:100 |
| *Enterococcus hirae* ATCC 10541 | $2,5 \times 10^8$ | R < 5 log | R > 5 ($2,67 \times 10^5$) | R > 5 ($2,27 \times 10^5$) |
| *Proteus vulgaris* ATCC 13315 | $2,4 \times 10^8$ | R < 5 log | R > 5 ($3,43 \times 10^5$) | R > 5 ($2,8 \times 10^5$) |
| *Pseudomonas aeruginosa* ATCC 15442 | $2,2 \times 10^8$ | R < 5 log | R > 5 ($8,9 \times 10^5$) | R > 5 ($4,93 \times 10^5$) |
| *Staphylococcus aureus* ATCC 6538 | $2,3 \times 10^8$ | R < 5 log | R > 5 ($4,60 \times 10^5$) | R > 5 ($3,54 \times 10^5$) |

| Test organism | N | Conditions: high level soiling | | |
|---|---|---|---|---|
| | | 1:250 | 1:80 | 1:100 |
| *Enterococcus hirae* ATCC 10541 | $2,5 \times 10^8$ | R < 5 log | R > 5 ($2,08 \times 10^5$) | R > 5 ($1,79 \times 10^5$) |
| *Proteus vulgaris* ATCC 13315 | $2,4 \times 10^8$ | R < 5 log | R > 5 ($2,53 \times 10^5$) | R > 5 ($2,4 \times 10^5$) |
| *Pseudomonas aeruginosa* ATCC 15442 | $2,2 \times 10^8$ | R < 5 log | R > 5 ($5,55 \times 10^5$) | R > 5 ($4,93 \times 10^5$) |
| *Staphylococcus aureus* ATCC 6538 | $2,3 \times 10^8$ | R < 5 log | R > 5 ($4,18 \times 10^5$) | R > 5 ($3,3 \times 10^5$) |

**Validation of membrane filtration method**

**Dilute the bacterial test suspensions with the diluent to obtain the bacterial count of** 6 x $10^2$ to 3 x $10^3$ cfu/ml.

For counting of the suspension prepare a $10^{-1}$ dilution with the diluent. Mix. Take a sample of 1,0 ml in duplicate and inoculate using the pour plate. Pipette each 1,0 ml in separate Petri dish and add 12 to 15 ml melted TSA, cooled to 45°C ± 1°C.

Incubate the plates at 36°C ± 1°C for 24 + 24 h.

Determine the number of cfu/ml in the test suspension ($N_v$) considering dilution factor $10^{-1}$ and volume 1 ml).

Colony counts per plate ($N_v$)

| Test organism | cfu/ml | $N_v$ |
|---|---|---|
| *Enterococcus hirae* ATCC 10541 | 110; 135 | 1,22 x $10^3$ |
| *Proteus vulgaris* ATCC 13315 | 150; 144 | 1,47 x $10^3$ |
| *Pseudomonas aeruginosa* ATCC 15442 | 110; 118 | 1,14 x $10^3$ |
| *Staphylococcus aureus* ATCC 6538 | 145; 161 | 1,53 x $10^3$ |

**Preparation of product test solution**

Prepare the product test solution at the highest strength (1:25) used in the test.

**Test for validation**

a) Validation of selected experimental conditions (verification of the absence of any lethal effect in the test conditions) (A).

Place in a sterile tube 1,0 ml of the interfering substance (high level soiling conditions) and 1,0 ml of the bacterial suspension (see $N_v$). Mix for a few seconds and leave in the water bath at 10°C ± 1°C for 2 min ± 10 s. At the end of this time, add 8,0 ml of hard water. Start the stopwatch at the beginning of the addition and mix for a few seconds.

Leave in the water bath at 10°C ± 1°C for 30 min ± 1° s.

At the end of contact time, sample in duplicate, 1,0 ml of the mixture and transfer into separate membrane filtration apparatus equipped with a membrane containing 50 ml of sterile distilled water. Filter and rinse with 50 ml of sterile distilled water and the transfer the membranes to the surface of two separate TSA plates.

Incubate the plates at 36°C ± 1°C for 24 + 24 h and calculate the number of cfu/ml in the experimental conditions validation (A).

b) Validation of filtration procedure (B)

Sample in duplicate 0,1 ml of the bacterial suspension (see $N_v$) and transfer into two separate membrane filtration apparatus equipped with a membrane containing 50 ml of sterile distilled water. Filter and rinse with 50 ml of sterile distilled water and then transfer the membranes to the surface of two separate TSA plates.

Incubate the plates at 36°C ± 1°C for 24 + 24 h and calculate the number of cfu/ml in the validation of filtration procedure (B).

c) Validation of the filtration method (counting of the bacteria on the membranes which have previously been in contact with the mixture of product and interference substance) (C)

Place in a sterile tube 1,0 ml of the interfering substance (high level soiling conditions) and 1,0 ml of the diluent and then starting the stopwatch, 8,0 ml of the of the product (1:100).

Mix and leave in the water bath at 10°C ± 1°C for 30 min ± 1° s.

At the end of contact time, sample in duplicate, 0,1 ml of the mixture and transfer into two separate membrane filtration apparatus equipped with a membrane containing 50 ml of sterile distilled water. Filter and rinse with 150 ml (max 500 ml) of sterile distilled water then cover the membranes with 50 ml of sterile distilled water and add 0,1 ml of bacterial suspension (see ($N_v$)

Filter and rinse with 50 ml of sterile distilled water and then transfer the membranes to the surface of two separate TSA plates.

Incubate the plates at 36°C ± 1°C for 24 + 24 h and calculate the number of cfu/ml in the validation of the filtration method (C).

**Verification of the methodology and validation of membrane filtration method at temperature tested (product AB12 dilution 1:100)**

| Test organism | $N_v$ | Validation of membrane filtration method and experimental conditions | | |
|---|---|---|---|---|
| | | Filtration procedure control (B) | Filtration test control (C) | Experimental conditions control (A) |
| *Enterococcus hirae* ATCC 10541 | $1,22 \times 10^3$ | 105; 108<br>$B = 1,06 \times 10^3$ | 95; 106<br>$C = 1,00 \times 10^3$ | 110; 113<br>$A = 1,11 \times 10^3$ |
| *Proteus vulgaris* ATCC 13315 | $1,47 \times 10^3$ | 142; 144<br>$B = 1,43 \times 10^3$ | 94; 101<br>$C = 9,75 \times 10^2$ | 135; 141<br>$A = 1,38 \times 10^3$ |
| *Pseudomonas aeruginosa* ATCC 15442 | $1,14 \times 10^3$ | 105; 114<br>$B = 1,09 \times 10^3$ | 88; 92<br>$C = 9,0 \times 10^2$ | 101; 106<br>$A = 1,03 \times 10^3$ |
| *Staphylococcus aureus* ATCC 6538 | $1,53 \times 10^3$ | 145; 139<br>$B = 1,42 \times 10^3$ | 111; 107<br>$B = 1,09 \times 10^3$ | 124; 129<br>$A = 1,26 \times 10^3$ |

N: number of cfu/ml of the bacterial test suspension; between $1,5x10^8$ and $5x10^8$

Nv: number of cfu/ml of the bacterial suspension; between $6x10^2$ and $3x10^3$

Vc: viable count (dilution $10^{-1}$)

A: number of cfu/ml of the experimental conditions validation: ≥ 0,05 times $N_v$

B: number of cfu/ml of the filtration validation: ≥ 0,05 times $N_v$

C: number of cfu/ml of the membrane test filtration validation: ≥ 0,5 times B.

**CONCLUSION**

The product code number VRKS demonstrates, diluted 1:100, a reduction more than $10^5$ in viability within 30 min at 10°C with the chosen interfering substance (low and high soiling level), under the conditions defined by the test UNI EN 1656: test organisms: *Enterococcus hirae, Proteus vulgaris, Pseudomonas aeruginosa* and *Staphylococcus aureus*.

**NOTE**

We received from Antec International Limited *via* Unitec (Milano) the Investigation into the effectiveness of the same product with code number VRKS executed by CCFRA Technology Limited, Chipping Champden – Gloucestershire GL55 6LD, UK, in accordance with the EN 1276:1997. The product supplied, at the use concentration (1%) possess bactericidal activity in 5 minutes at 10°C under clean and dirty conditions for the reference strains: *Escherichia coli, Enterococcus hirae, Pseudomonas aeruginosa, Staphilococcus aureus* **plus** *Listeria monocytogenes, Salmonella typhimurium, Yersinia enterocolitica*.

**LOCALITY AND CONSERVATION OF THE ROW DATA**

The program of the study and a copy of the same shall be conserved in the archives of the Centro di Saggio for a period of 5 years from the date of this final report

# ALLEGATO N. 1

**Rapporto di prova 37/01 del laboratorio IMS s.r.l. di Pomezia**

***Prove quantitative in sospensione per la valutazione dell'attività battericida di disinfettanti chimici ed antisettici utilizzati in campo veterinario (Norma EN 1657)***

**VIRKON S** - ***Conferma della autorizzazione***

**ALLEGATO N. 3**

**Rapporto di prova 37/02 del laboratorio IMS s.r.l. di Pomezia**

***Prove quantitative in sospensione per la valutazione dell'attività fungicida di disinfettanti chimici ed antisettici utilizzati in campo veterinario (NORMA en 1657)***

**VIRKON S** - ***Conferma della autorizzazione***