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Antimicrobial wound dressings - Requirements and test method

Pansements antimicrobiens - Exigences et méthode d'essai

Antimikrobielle Wundauflagen - Anforderungen und Prüfverfahren

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EUROPEAN COMMITTEE FOR STANDARDIZATION
COMITÉ EUROPÉEN DE NORMALISATION
EUROPÄISCHES KOMITEE FÜR NORMUNG

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European foreword

This document (prEN 17854:2022) has been prepared by Technical Committee CEN/TC 205 “Non-active medical devices”, the secretariat of which is held by DIN.

This document is currently submitted to the CEN Enquiry.

Introduction

This document describes a test method for establishing whether a wound dressing exerts antimicrobial activity.

The laboratory test attempts to simulate conditions of application, through the use of appropriate test fluids, temperature, organisms, and contact times reflecting the parameters found in clinical situations. Conditions which may influence the action of wound dressings having antimicrobial properties have been included.

The conditions are intended to cover general purposes and to allow comparison between laboratories and product types.

This edition of the document is considered to be most suited for dressings that have been tested as part of the inter-laboratory comparisons, i.e. that have an active antimicrobial agent incorporated and with at least a small amount of absorbent capacity. No data has currently been generated on other types of dressing (e.g. film, non-absorbing, multi-layered, adhesive, bacteria-binding dressing, etc.).

1 Scope

This document specifies minimum requirements and a test method for the antimicrobial (microbicidal or microbistatic) activity of wound dressing products. It applies to all wound dressing products that specifically claim antimicrobial activity according to this document.

2 Normative references

The following documents are referred to in the text in such a way that some or all of their content constitutes requirements of this document. For dated references, only the edition cited applies. For undated references, the latest edition of the referenced document (including any amendments) applies.

EN 12353, *Chemical disinfectants and antiseptics - Preservation of test organisms used for the determination of bactericidal (including Legionella), mycobactericidal, sporicidal, fungicidal and virucidal (including bacteriophages) activity*

3 Terms, definitions, symbols and abbreviated terms

3.1 Terms and definitions

For the purposes of this document, the following terms and definitions apply.

ISO and IEC maintain terminological databases for use in standardization at the following addresses:

— IEC Electropedia: available at https://www.electropedia.org/

— ISO Online browsing platform: available at https://www.iso.org/obp

3.1.1
antimicrobial dressing
wound dressing which can be shown to exert microbicidal or microbistatic properties

3.1.2
antimicrobial activity
A
capability of a wound dressing to either inhibit the growth or produce a reduction in the number of viable cells of relevant test organisms under defined conditions, including viable bacterial cells and/or viable vegetative yeast cells

3.1.3
CFU
colony forming unit(s)

3.1.4
microbicidal
capability of the dressing to produce at least a 3 log reduction in the number of viable cells from the challenge organisms when tested under the conditions in Clause 5

3.1.5
microbistatic
capability of the dressing to at least prevent further growth of the initial inoculum when tested under the conditions in Clause 5

3.1.6
negative control dressing
wound dressing which is the same dressing as the dressing to be tested but without the antimicrobial treatment

Note 1 to entry: If no similar non-medicated dressing is available, then a sterile gauze swab shall be used.

3.1.7
neutralizer
chemical formulation to stop the antimicrobial effect of antimicrobial agents

3.1.8
plate count method
method in which the number of viable microorganisms present after incubation is calculated by counting the number of CFU

3.1.9
plate factor
factor used in CFU calculations which accounts for the volume of suspensions plated out onto agar plates

Note 1 to entry: This factor differs depending on choice of pour plates or spread plates.

3.1.10
saturation volume
SV
maximum volume of fluid absorbed by the dressing when tested according to the method in 5.5 of this document

3.1.11
simulated wound fluid
SWF
test medium intended to simulate wound exudate, for suspension of test organisms prior to exposure to test sample

3.1.12
test dressing
wound dressing which is tested to assess its antimicrobial activity

3.1.13
working volume
WV
volume of SWF added to the test or control dressing during the test, determined as 80 % of the saturation volume (ml)

3.2 Symbols and abbreviated terms

For the purposes of this document, the following symbols and abbreviated terms apply.

A	Antimicrobial activity
C_0	CFU value at T = 0 h for negative control dressing
C_{24}	CFU value at T = 24 h for negative control dressing
C_C	Microbial concentration of *INOC C*
C_s	Microbial concentration per sample (CFU / sample) used to calculate *INOC* C (in Table 5)
C_T	Viable counts per dressing sample
C_V	Viable counts per ml
DF	Dilution factor
h	Hour
INITIAL STOCK	Microbial suspension in MRD harvested from agar sub-culture
INOC C	Inoculum for negative control dressing (prepared from STOCK B and calculated volume of uninoculated SWF equating to WV_C – 0,5 ml)
INOC T	Inoculum for test dressing (prepared from *STOCK B* and calculated volume of uninoculated SWF equating to WV_T – 0,5 ml)
LOD	Limit of detection
Log C_0	is the average Log value for the number of organisms obtained from three negative control dressing samples immediately after inoculation
Log T_{24}	is the average Log value for the number of organisms obtained from three test dressing samples after incubation for 24 h
min	Minutes
N	Average number of CFU on P_1 and P_2
N_0	Undiluted test suspension
NE	Neutralization effectiveness (%)
N_{EFF}	Average Neutralizer efficacy in neutralization validation (CFU/ml)
NT	Neutralization toxicity (%)
N_{TOX}	Average Neutralizer toxicity in neutralization validation (CFU/ml)
N_{VIAB}	Average Test organism viability in neutralization validation (CFU/ml)
P_1	The number of organisms on plate 1 of duplicate agar plates (CFU)
P_2	The number of organisms on plate 2 of duplicate agar plates (CFU)
PF	Plate factor

s	Second
STOCK A	Microbial suspension of *INITIAL STOCK* diluted in MRD to contain 3×10^8 CFU/ml to 1×10^9 CFU/ml, used to prepare *STOCK B* and *STOCK N*
STOCK B	Microbial suspension of *STOCK A* diluted in SWF to contain 3×10^6 CFU/ml to 1×10^7 CFU/ml
STOCK N	Microbial suspension of *STOCK A* diluted in MRD to contain $1{,}5 \times 10^3$ CFU/ml to $5{,}0 \times 10^3$ CFU/ml, used in Neutralization Validation when using 1 ml pour plates
SV	Saturation volume
SV_A	Average saturation volume of five replicates (g)
SV_{HIGH}	Highest percentage saturation volume of five replicates (%)
SV_{LOW}	Lowest percentage saturation volume of five replicates (%)
SV_{MAX}	Highest measured saturation volume of five replicates (g)
SV_{MIN}	Lowest measured saturation volume of five replicates (g)
SV_{SPREAD}	Percentage spread for the five replicates (%)
T_{24}	CFU value at T = 24 h for test dressing
V_N	Neutralizer Volume
V_S	Volume sampled from the paddle blender bag when preparing serial decimal dilutions in 5.8.2.4 (ml)
V_T	Test volume = Neutralizer volume (V_N) + Working Volume (WV_T or WV_c) (ml)
W_0	Average dressing weight at T = 0 h (g)
W_{Te}	The average fully saturated dressing weight (g), as determined by the weight not changing between two time points by more than 5 %
W_{Tv}	Dressing weight following saturation in SWF at each time point as applicable (g)
WV	Working volume (3.1.13) – the volume of SWF added to the test or control dressing during the test, determined as 80 % of the saturation volume (ml)
WV_C	Working volume (WV) added to the negative control dressing (ml)
WV_T	Working volume (WV) added to the test dressing (ml)

4 Requirements

4.1 Documentation and training

Laboratories performing the test in this document should be operating under an appropriate quality management system (such as EN ISO 13485 [1], EN ISO/IEC 17025 [2] or similar). Therefore, ensuring that suitable records are retained by the test laboratory to allow full traceability of all raw data contributing to the results in the test report (5.9) and that competence in microbiological testing has been established.

4.2 Microbicidal dressings

When tested using the test method described in this document, antimicrobial dressings shall demonstrate an antimicrobial activity of at least a 3 log reduction in activity in a valid test (5.8.5) at the mandatory contact time of 24 h (T = 24 h) and against all three test organisms (5.3.1).

NOTE 1 Log values in this document refer to Log_{10}.

NOTE 2 Rationale for microbicidal requirements is given in Annex D.

4.3 Microbistatic dressings

When tested using the test method described in this document, microbistatic dressings shall demonstrate an antimicrobial activity that prevents further growth of the initial inoculum in a valid test (5.8.5) at the mandatory contact time of 24 h (T = 24 h) and against all three test organisms (5.3.1). This means at least a 0 log reduction is obtained, which represents no increase in test organism numbers at the mandatory contact time of 24 h (T = 24 h) against all three test organisms (5.3.1).

4.4 Performance table

Table 1 shows the performance requirements for antimicrobial dressings to be classified as microbicidal or microbistatic.

Table 1 — Performance requirements for antimicrobial dressings

Microbicidal	Microbistatic
$A \geq 3{,}0$ against all 3 test organisms	$A \geq 0{,}0$ against all 3 test organisms
A = Antimicrobial activity (5.8.3.5)	

If a test dressing does not meet either a microbicidal or microbistatic requirement then it is non-antimicrobial according to this document.

The test report (5.9) shall be supplied where claims of compliance with this document are made.

If a manufacturer makes a claim for antimicrobial activity for contact times in addition to the mandatory contact time of 24 h (T = 24 h) then those additional time points may be tested using this document and the results shall be included in the test report (5.9) and made available on request.

The test method in this document may be used with additional test organisms, including molds, provided that appropriate neutralization validation has been performed. If the results using such additional test organisms are made publicly available, then the neutralization validation shall be made available on request.

NOTE Example tables are given in Annex G.

5 Test method

5.1 Principle

A test suspension of bacteria or yeast in a solution of simulated wound fluid is inoculated into a sample of a wound dressing (test sample). The volume of test suspension added is determined by calculating the saturation volume of the test sample prior to inoculation. The test sample is maintained at a specified temperature for a defined contact time, then transferred to a previously validated neutralization medium so that the action of the antimicrobial agent is neutralized. The number of surviving bacteria or yeast, which can be recovered from the test sample, is then quantitatively determined, and compared to the number of bacteria or yeast in a negative control at start of the test.

The negative control sample dressing shall be treated in the same manner in place of the test sample during the whole test period.

NOTE Rationale for chosen test parameters is given in Annex D.

Any permitted deviations to this test method shall be suitably validated, documented in the report and their use justified.

5.2 General conditions

5.2.1 Volume accuracy

Volumes shall be measured with calibrated pipettes. Unless otherwise specified, all volumes are expected to be accurate to ±5 %.

5.2.2 Agar plates

This document is written with the intention that 1 ml agar pour plates are used for enumeration. However, it is known that some laboratories may be limited to the use of spread plates for enumeration. The change in volume when spread plates are used requires accounting for at various points in the method. Therefore, throughout this document instructions for the adjustments required when using spread plates have been given and users of this document should ensure the required adjustments are carefully followed.

NOTE The use of a very low volume for spread plates will reduce the sensitivity of the test and this may affect the ability to ability to calculate the required 3 log reduction. Therefore, the use of 1 ml pour plates at all times is encouraged.

5.3 Materials and reagents

5.3.1 Test organism strains

5.3.1.1 Storage of organisms

The organisms shall be stored in accordance with the supplier's recommendations or EN 12353.

The identification and origin (culture collection) of the organisms as well as the laboratory storage method shall be recorded.

National collection test organism strains equivalent to those listed in 5.3.1.2 and 5.3.1.3 may be used; some common alternative references in other national collections are given in Annex A.

5.3.1.2 Bacteria

The following two strains of bacteria shall be evaluated:

Pseudomonas aeruginosa ATCC 9027

Staphylococcus aureus ATCC 6538

5.3.1.3 Yeast

The following strain of yeast shall be evaluated:

Candida albicans ATCC 10231

5.3.1.4 Additional test organism strains

Additional test organism strains to those listed in 5.3.1.2 and 5.3.1.3 may be used for evaluation. Their suitability for producing inocula of sufficient concentration shall be verified prior to use.

If the additional strains are not classified in a reference collection (e.g. clinical isolates), their identification characteristics should be stated in the report and they should be held by the testing laboratory for a minimum of five years to allow repeat testing if necessary.

5.3.2 Reagents and culture media

5.3.2.1 General

Reagents used in tests shall be of analytical quality and/or suited for microbiological purposes. Dehydrated or ready-prepared products available on the commercial market are recommended for use in preparing the culture media. The manufacturer's instructions for the preparation, sterilization and storage of culture media shall be strictly followed. Unless otherwise specified, all reagents shall be equilibrated at ambient temperature (15 °C to 25 °C) before use.

Alternative media to those listed may be used, provided they have been suitably validated for use. The use of alternative agar technologies, e.g. Petrifilm™ or Compact Dry™[1] plates, is also acceptable, provided their use has been suitably validated and used to perform Neutralization Validation (5.7). Any alternative media or media technologies shall be documented in the report as a deviation (5.9).

Substitution of FCS in the SWF (5.3.2.5) is not recommended (see Annex D, D.8).

5.3.2.2 Water

Purified water shall be used for the preparation of culture media, i.e. distilled, demineralized, deionized or produced by reverse osmosis, or of equivalent quality free from substances likely to inhibit or influence the growth of the microorganisms under the test conditions, e.g. traces of chlorine, traces of ammonia and traces of metal ions.

NOTE See EN ISO 11133 [3] for more information on culture media preparation.

5.3.2.3 Tryptone Soya Broth (TSB)

For the maintenance of bacteria and performance of viable counts.

Tryptone, pancreatic digest of casein	15,0 g
Soya peptone, papaic digest of soya	5,0 g
NaCl	5,0 g
Water	to 1 000 ml

Sterilize in the autoclave. After sterilization, the pH shall be between 7,1 to 7,5.

5.3.2.4 Tryptone Soya Agar (TSA)

For the maintenance of bacteria and performance of viable counts.

Tryptone, pancreatic digest of casein	15,0 g
Soya peptone, papaic digest of soya	5,0 g
NaCl	5,0 g
Agar	15,0 g
Water	to 1 000 ml

[1] Petrifilm™ and Compact Dry™ are examples of alternative agar products available commercially. This information is given for the convenience of the users of this document and does not constitute an endorsement by CEN or CENELEC of these products.

Sterilize in the autoclave. After sterilization, the pH shall be between 7,1 to 7,5.

5.3.2.5 Simulated Wound Fluid (SWF)

For suspension of test organisms prior to exposure to test sample.

Maximum Recovery Diluent (5.3.2.6)	50 %
Fetal Calf Serum (sterile, heat inactivated*)	50 %

Mix MRD and fetal calf serum under aseptic conditions.

NOTE *Complement inactivated by the supplier or by the laboratory prior to use.

5.3.2.6 Maximum recovery diluent (MRD)

For preparation of SWF and test organism suspensions.

Peptone	1,0 g
NaCl	8,5 g
Water	to 1 000 ml

Sterilize in the autoclave. After sterilization, the pH shall be between 6,8 to 7,2.

5.3.2.7 Sabouraud broth (SAB)

For the maintenance of yeast and performance of viable counts.

Peptone Neutral Bact. Peptone	10 g
Distilled water	1 000 ml

Heat to boiling to dissolve the peptone completely. Then add:

Glucose puriss (dextrose)	20 g

Dissolve the glucose by hand as soon as possible in the hot, but not boiling agar, and mix.

Sterilize in the autoclave. After sterilization, the pH shall be between 5,8 to 6,0.

5.3.2.8 Sabouraud agar (SAA)

For the maintenance of yeast and performance of viable counts.

Peptone Neutral Bact. Peptone	10 g
Agar bacteriological	18 g
Distilled water	1 000 ml

Heat to boiling to dissolve the peptone completely. Then add:

Glucose puriss (dextrose)	20 g

Dissolve the glucose by hand as soon as possible in the hot, but not boiling agar, and mix.

Sterilize in the autoclave. After sterilization, the pH shall be between 5,8 to 6,0.

5.3.2.9 Neutralizer

The neutralizing solution shall be validated for the active substance in the product under test in accordance with 5.7. The neutralizer shall be sterile prior to use.

NOTE Information on neutralizers that have been found to be suitable for some categories of products is given in Annex C.

5.3.3 Test apparatus

Usual microbiological laboratory apparatus shall be required and in particular the following:

5.3.3.1 Spectrophotometer, capable of measuring at a 600 nm to 660 nm wavelength, or McFarland's nephelometer.

5.3.3.2 Incubators, capable of maintaining the following ranges: 20 °C to 25 °C, 30 °C to 34 °C and 30 °C to 35 °C.

5.3.3.3 Paddle blender, small size capable of handling volume up to 100 ml (e.g. Stomacher 80, Biomaster or Neutec Masticator 2 Compact model – 80 ml)[2].

5.3.3.4 Sterile paddle blender bags.

5.3.3.5 Mixer, electromechanical agitator (e.g. Vortex® mixer**Error! Bookmark not defined.**).

5.3.3.6 Various sterile containers, test tubes, bottles and flasks of suitable capacity including 60 ml.

5.3.3.7 Sterile Petri dishes, 55 mm diameter (height 16 mm) and 90 mm diameter.

5.3.3.8 Forceps, sterile.

5.3.3.9 pH meter, suitably calibrated and capable of measuring the pH with an accuracy of ±0,1 pH units at 19 °C to 21 °C.

5.3.3.10 Autoclave, capable of being maintained at 121 °C to 124 °C for a minimum holding time of 15 min.

5.3.3.11 Pipettes, of nominal capacities 10 ml, 1 ml and 0,1 ml. Automatic pipettes may be used.

5.3.3.12 Cutting equipment, to provide dressing samples as specified in 5.4.3 (e.g. biopsy punch or equivalent cutter).

5.3.3.13 Timer or stopwatch, calibrated.

5.4 Preparation of test and negative control dressings

5.4.1 Details of the negative control and test dressings to be tested shall be recorded per the requirements of the test report (5.9).

5.4.2 Negative control dressings (3.1.6) and test dressings (3.1.12) shall be prepared as detailed in 5.4.3. If a negative control dressing is not available, then use a non-medicated dressing from the same product group preferably from the same manufacturer. If no similar non-medicated dressing is available, then a sterile gauze swab shall be used. The gauze shall be cut to the same size as the test dressing and layered such that the weight allows the same saturation volume as the test dressing (see 5.5) to be added.

[2] Stomacher 80 Biomaster, Neutec Masticator 2 Compact model – 80 ml and Vortex® mixer are examples of suitable products available commercially. This information is given for the convenience of the users of this document and does not constitute an endorsement by CEN or CENELEC of these products.

5.4.3 Cut samples of test and control dressings with an area equivalent to 284 mm^2 to 346 mm^2 under aseptic conditions by using a sterile cutter. For example, a 20 mm diameter circular sample has an area of 314,2 mm^2.

5.5 Calculation of saturation volume and working volume[3]

5.5.1 Saturation volume (*SV*) shall be determined for both the test dressing and negative control dressing. Determination of the saturation volume (*SV*) shall be carried out at ambient temperature (15 °C to 25 °C). Timings stated in this clause shall be accurate to ±30 s.

NOTE Saturation volume is initially determined by weight (g) then converted to volume (ml) when calculating the working volume (5.5.13).

5.5.2 Weigh five replicates of each test and negative control dressing in empty 55 mm petri dishes and record weights to two decimal places (W_0).

5.5.3 Add aliquots of 12 ml SWF (an excess of SWF is required when determining *SV*) into ten Petri dishes with diameter of 55 mm in diameter. Allow the SWF to adjust to ambient temperature (15 °C to 25 °C).

NOTE The volumes do not have to be exact.

5.5.4 After being weighed, transfer each sample into the Petri dish containing SWF using forceps (5.5.3). Test and negative control dressings that have a wound contact surface shall be placed with the wound contact surface facing downwards onto the bottom of the petri dish. Leave on the bench for 30 min.

5.5.5 Gently remove dressing from the SWF using forceps without squeezing and allow any excess fluid to drain for 5 s to 10 s. Thereafter, tap the dressing once on the side of the Petri dish to remove any drops of SWF. Transfer the dressing sample to an empty petri dish that is already on a balance that has been tared, and weigh. Record weights of all replicate dressing samples to two decimal places. These weights are denoted W_{Tv}. Tare the balance with the Petri dish; it is the weight of the dressing that should be recorded. Residual fluid will thereby have no effect on the saturation volume.

5.5.6 After weighing, return replicate to the same SWF-containing Petri dish as before (5.5.4) and continue to incubate at ambient temperature (15 °C to 25 °C).

5.5.7 Repeat the weighing of the dressings at 1 h and further 30 min intervals.

5.5.8 Use the weights determined at the first time point where there was ≤ 5 % change in weight from previous reading, this weight is denoted W_{Te}. Dressings are considered fully saturated when weight does not change between two time points by more than 5 %. An example of weights and calculations to determine W_{Te} is given in Table 2.

[3] For a graphical representation of Calculation of Saturation Volume and Working Volume, see Figure F.1.

Table 2 — Example calculation of W_{Tes}

Time point	Dressing weights W_{Tv} g	Average dressing weights g	Average change %
0 h 0 min (5.5.2)	0,132, 0,133, 0,128, 0,132, 0,140	0,133 (W_0)	N/A
0 h 30 min	2,357, 2,066, 2,038, 1,556, 1,791	1,962 (W_{Te})[a]	N/A
1 h 00 min	2,322, 2,044, 2,053, 1,595, 1,851	1,973	0,58
1 h 30 min	2,376, 2,041, 2,030, 1,630, 1,834	1,982	0,47
2 h 00 min	2,341, 2,090, 2,059, 1,649, 1,848	1,997	0,77

[a] In this example, although weights (W_{Tv}) were taken every 30 min to 2 h, the weights did not change by more than 5 % between 30 min and 60 min. Therefore, the weight when the dressing was considered fully saturated (W_{Te}) was the average weight at 30 min – 1,962 g.

NOTE It is expected that full saturation will occur within 4 h based upon the experience of the working group during development of this document.

5.5.9 Use the following formula to calculate the SV for each test and negative control dressings:

$$SV = W_{Te} - W_0$$

where

SV is the saturation volume (g);

W_0 is the average dressing weight at T = 0 h (g);

W_{Te} is the average fully saturated dressing weight (g), as determined by the weight not changing between two time points by more than 5 %.

5.5.10 Calculate the average saturation volume (SV_A) for the test and negative control dressings.

5.5.11 Use the following formulae to calculate the spread for the replicates:

$$SV_{LOW} = \frac{SV_A - SV_{MIN}}{SV_A} \times 100$$

$$SV_{HIGH} = \frac{SV_{MAX} - SV_A}{SV_A} \times 100$$

$$SV_{SPREAD} = SV_{HIGH} - SV_{LOW}$$

where

SV_A is the average saturation volume of five replicates (g);

SV_{MIN} is the lowest measured saturation volume of five replicates (g);

SV_{MAX} is the highest measured saturation volume of five replicates (g);

SV_{SPREAD} is the percentage spread for the five replicates (%);

SV_{LOW} is the lowest percentage saturation volume of five replicates (%);

SV_{HIGH} is the highest percentage saturation volume of five replicates (%).

5.5.12 SV_{SPREAD} is acceptable if it is ≤ 20 %. If SV_{SPREAD} is > 20 % then the test shall be repeated a further two times to confirm the result. If SV_{SPREAD} is confirmed to be > 20 % (i.e. variable) then the use of this value shall be documented and justified in the report.

5.5.13 Convert SV_A for both negative control and test dressings from weight (g) to volume (ml), assuming equivalency between 1 g = 1 ml.

5.5.14 Calculate the *WORKING VOLUME* (*WV*) to be added to samples (WV_T for test dressings or WV_C for negative control dressings) when exposing dressings to test organisms (5.8.1).

$$WV_C = 0,8 \times SV_A$$

$$WV_T = 0,8 \times SV_A$$

where

SV_A is average saturation volume of five replicates (ml);

WV is working volume (3.1.13) – the volume of SWF added to the test or control dressing during the test, determined as 80 % of the saturation volume (ml);

WV_C is working volume (*WV*) added to the negative control dressing (ml);

WV_T is working volume (*WV*) added to the test dressing (ml).

5.5.15 Record the WV_C and WV_T results, rounded to one decimal place.

5.6 Preparation of test organism suspensions

5.6.1 Bacteria

5.6.1.1 The test organisms and their stock cultures shall be prepared and kept in accordance with EN 12353.

5.6.1.2 Prepare a subculture from the stock culture by streaking onto TSA slopes (also known as slants) or TSA plates and incubate at 30 °C to 35 °C.

5.6.1.3 After 18 h to 24 h prepare a second subculture from the first subculture in the same way and incubate for 18 h to 24 h.

5.6.1.4 From this second subculture, a third subculture may be produced in the same way. The second and (if produced) third subculture are the working cultures.

If it is not possible to prepare the second subculture on a particular day, a 48 h subculture may be used, provided that the subculture has been kept in the incubator at 30 °C to 35 °C during the 48 h period.

Only second or third subcultures shall be used.

5.6.2 Yeast

5.6.2.1 The test organisms and their stock cultures shall be prepared and kept in accordance with EN 12353.

5.6.2.2 Prepare a subculture from the stock culture by streaking onto SAA slopes (also known as slants) or SAA plates and incubate at 30 °C to 35 °C.

5.6.2.3 After 24 h to 48 h, prepare a second subculture from the first subculture in the same way and incubate for 24 h to 48 h.

NOTE *C. albicans* ATCC 10231 usually grows sufficiently well within 24 h to 48 h at 30 °C to 35 °C.

5.6.2.4 From this second subculture, a third subculture may be produced in the same way. The second and (if produced) third subculture are the working cultures.

If it is not possible to prepare the second subculture on a particular day, a 72 h subculture may be used, provided that the subculture has been kept in the incubator at 30 °C to 35 °C during the 72 h period.

Only second or third subcultures shall be used.

5.6.2.5 The conditions listed in 5.6.1 and 5.6.2 may not be appropriate for any additional organisms tested (5.3.1.4), the most suitable conditions for growth and preparation for the additional test organisms should be identified and used.

5.6.3 Preparation of *STOCK A*[4]

5.6.3.1 Take 10 ml of MRD and place in a 100 ml flask with 5 g of sterile glass beads.

NOTE The volume does not have to be exact.

5.6.3.2 Take the working culture produced in 5.6.1.4 or 5.6.2.4 and transfer loopfuls of the cells into the MRD. The cells shall be suspended in the diluent by rubbing the loop against the wet wall of the container to dislodge the cells before immersing in the diluent.

5.6.3.3 Shake the container for 3 min using a mechanical shaker then transfer the suspension to a suitable sterile bottle or tube. This is the *INITIAL STOCK*.

5.6.3.4 Adjust the number of cells in the *INITIAL STOCK* to 3×10^8 CFU/ml to 1×10^9 CFU/ml using MRD estimating the number of CFU by any suitable means. This suspension is called *STOCK A*.

NOTE Suitable means could include using a colorimeter to measure optical density and comparing to previously generated standard curves or use of McFarland standards.

5.6.3.5 Maintain *STOCK A* at ambient temperature (15 °C to 25 °C) and use within 2 h of preparation.

[4] For a graphical representation of Preparation of *STOCK A*, see Figure F.3 in Annex F.

5.7 Neutralization validation[5]

Validation of the neutralizing solution is required to ensure the activity of the antimicrobial agent can be effectively inactivated, or quenched, after the contact time and that the neutralizing solution does not affect the viability of any test organisms remaining.

NOTE 1 It is not normally required to repeat this step (5.7) unless the product has changed. Previous neutralization validation data can be acceptable and used in the report if the product hasn't changed since it was generated.

NOTE 2 Justification for neutralization validation is given in Annex B.

5.7.1 Preparation of inoculum

5.7.1.1 Dilute *STOCK A* in MRD to 1,5 × 10^3 CFU/ml to 5,0 × 10^3 CFU/ml, this suspension is called *STOCK N*. This means that the final concentration within each neutralizer validation suspension contains 30 CFU/ml to 100 CFU/ml.

5.7.1.2 To check if the concentration used was in the correct range (1,5 × 10^3 CFU/ml to 5,0 × 10^3 CFU/ml), perform a count of *STOCK N* prepared in 5.7.1.1 using standard microbiological techniques on agar.

5.7.1.3 If spread plates are used in place of pour plates, then *STOCK N* shall be adjusted to ensure that the counts on the plates will remain in the range of 30 CFU/ml to 100 CFU/ml. This is done by adjusting *STOCK N* by the plate factor (the spread plate volume). See Table 3 for adjustments for two example volumes of spread plates (0,1 ml and 0,5 ml).

Table 3 — Example calculations to adjust *STOCK N* when using spread plates

STOCK N for 1 ml pour plate	***STOCK N*** adjustment calculation for 0,1 ml spread plate	***STOCK N*** adjustment for 0,5 ml spread plate
1,5 × 10^3 CFU/ml	$\frac{1,5\times10^3}{0,1}=1,5\times10^4$	$\frac{1,5\times10^3}{0,5}=3,0\times10^3$
5,0 × 10^3 CFU/ml	$\frac{5,0\times10^3}{0,1}=5,0\times10^4$	$\frac{5,0\times10^3}{0,5}=1,0\times10^4$
NOTE 1 In these examples, when using 0,1 ml spread plates the adjusted range is 1,5 × 10^4 CFU/ml to 5,0 × 10^4 CFU/ml and for 0,5 ml spread plates it is 3,0 × 10^3 CFU/ml to 1,0 × 10^4 CFU/ml. NOTE 2 When conducting neutralization validation using spread plates if *STOCK N* was not adjusted, as in this example for 0,1 ml spread plates, then counts in the range 3 CFU/ml to 10 CFU/ml would be obtained. It is not good microbiological practice to allow the use of such a low inoculum for neutralization validation.		

5.7.2 Neutralizer toxicity

5.7.2.1 Perform neutralizer toxicity (N_{TOX}) in triplicate for each test organism to demonstrate that the chosen neutralizer does not have any inhibitory effect of the test organisms used in the test.

5.7.2.2 Transfer aliquots of 4,4 ml of the neutralizer to test tubes.

5.7.2.3 Inoculate each test tube with 100 µl of *STOCK N*.

[5] For a graphical representation of neutralization validation, see Figure F.4 in Annex F.

5.7.2.4 Add 0,5 ml of SWF to each test tube of the test organism and mix well. The concentration in the test tubes shall be 30 CFU/ml to 100 CFU/ml.

5.7.2.5 Allow the suspensions to stand at ambient temperature (15 °C to 25 °C) for the longest time period that would be representative of the time that the suspensions will stand before being serially diluted and plated out during testing (5.8.2).

NOTE Unless prior knowledge and experience in the use of the chosen neutralizer is known, a separate exercise can be performed to determine optimal standing time for effective neutralization and absence of toxicity prior to performing the test.

5.7.2.6 After the exposure time, transfer 1 ml volumes of the suspension to duplicate TSA or SAA pour plates. If spread plates are used in place of 1 ml pour plates, ensure *STOCK N* has been adjusted (5.7.1.3).

5.7.2.7 Following incubation at 30 °C to 35 °C for 24 h to 72 h, count the number of colonies on each agar plate and calculate *N* (see 5.7.5.1).

5.7.3 Test organism viability

5.7.3.1 Perform test organism viability (N_{VIAB}) in triplicate for each test organism to determine the population of each test organism in the neutralization assay.

5.7.3.2 Transfer aliquots of 4,4 ml of MRD to test tubes.

5.7.3.3 Inoculate each test tube with 100 µl of *STOCK N*.

5.7.3.4 Add 0,5 ml of SWF to each test tube. The concentration in each test tube shall be 30 CFU/ml to 100 CFU/ml of the test organism. Mix content.

5.7.3.5 Transfer 1 ml volumes of the suspension to duplicate TSA or SAA pour plates. If spread plates are used in place of 1 ml pour plates, ensure *STOCK N* has been adjusted (5.7.1.3).

5.7.3.6 Following incubation at 30 °C to 35 °C for 24 h to 72 h, count the number of colonies on each agar plate and calculate *N* (see 5.7.5.1).

5.7.4 Neutralizer efficacy

5.7.4.1 Perform neutralizer efficacy (N_{EFF}) in triplicate for each test organism to demonstrate that the neutralizer is effective at inactivating or quenching the microbicidal properties of the antimicrobial agent.

5.7.4.2 Transfer a suitable volume of neutralizer (V_N) to sterile paddle blender bags.

NOTE Due to the wide variety of antimicrobial wound dressings, the volume of neutralizer required cannot be pre-determined as it will depend upon the dressing being tested. A volume of 20 ml Dey-Engley (D/E) neutralizing broth was found to be suitable for silver and PHMB dressings in inter laboratory tests undertaken during the development of this document.

5.7.4.3 Inoculate the neutralizer in each paddle blender bag with *STOCK N* at a volume which is $^1/_{50}$ of the required neutralizer volume.

5.7.4.4 Transfer one test dressing sample to each paddle blender bag containing the neutralizer and the challenge organism. Process in the paddle blender for an appropriate time and speed, then allow the suspension to stand for an appropriate time at ambient temperature (15 °C to 25 °C). Document the paddle blender parameters (time and speed) and the suspension standing time used. If an alternative recovery method during testing is used (5.8.2.2), it shall be used in place of the paddle blender to process the samples.

NOTE 1 Unless prior knowledge and experience in the use of the chosen neutralizer is known, a separate exercise can be performed to determine optimal standing time for effective neutralization prior to performing the test.

NOTE 2 The experience of the Working Group is that a paddle blender is the most appropriate method for recovery of organisms from wound dressings. See Rationale (D.15) for further information.

5.7.4.5 Transfer 1 ml volumes of the suspension from 5.7.4.4 to duplicate TSA or SAA pour plates. If spread plates are used in place of 1 ml pour plates, ensure *STOCK N* has been adjusted (5.7.1.3).

5.7.4.6 Following incubation at 30 °C to 35 °C for 24 h to 72 h, count the number of colonies on each agar plate and calculate *N* (see 5.7.5.1)

5.7.5 Interpretation of data

5.7.5.1 Apply the following formula to calculate *N* resulting from each replicate:

$$N = \frac{P_1 + P_2}{2}$$

where

N is the average number of P_1 and P_2 (CFU);

P_1 is the number of organisms on plate 1 of duplicate agar plates (CFU);

P_2 is the number of organisms on plate 2 of duplicate agar plates (CFU).

NOTE The value for *N* in 5.7.5.1 is calculated in CFU because the volume plated out using either pour plates or spread plates is irrelevant for the purposes of this calculation as *STOCK N* will have already been adjusted if spread plates are used (5.7.1.3).

5.7.5.2 Calculate the average of *N* for the three replicates, rounding up to the nearest whole CFU, for each of the following:

a) neutralizer toxicity N_{TOX} (carried out in 5.7.2);

b) test organism viability N_{VIAB} (carried out in 5.7.3);

c) neutralizer efficacy N_{EFF} (carried out in 5.7.4).

5.7.5.3 Use the values to determine neutralization effectiveness (5.7.5.4) and neutralization toxicity (5.7.5.5).

5.7.5.4 Neutralization effectiveness

5.7.5.4.1 Use the following formula to calculate neutralization effectiveness (*NE*) from the average number of surviving test organisms:

$$NE = \frac{N_{\mathrm{EFF}}}{N_{\mathrm{VIAB}}} \times 100$$

where

NE is the neutralization effectiveness (%);

N_{EFF} is the average neutralizer efficacy in neutralization validation (CFU);

N_{VIAB} is the average test organism viability in neutralization validation (CFU).

5.7.5.4.2 Neutralization is considered effective if *NE* is 50 % to 200 %.

5.7.5.5 Neutralization toxicity

5.7.5.5.1 Use the following formula to calculate the neutralization toxicity (*NT*) from the average number of surviving test organisms:

$$NT = \frac{N_{\mathrm{TOX}}}{N_{\mathrm{VIAB}}} \times 100$$

where

NT is the neutralization toxicity (%);

N_{TOX} is the average neutralizer toxicity in neutralization validation (CFU);

N_{VIAB} is the average test organism viability in neutralization (CFU).

5.7.5.5.2 The neutralization medium is considered non-toxic if *NT* is 50 % to 200 %.

5.7.5.6 Example calculations

5.7.5.6.1 An example set of calculations for neutralization validation is shown in Table 4.

Table 4 — Example set of data and calculations of *NE* and *NT*

Group	Replicate	P_1	P_2	N	Average N	*NE*	*NT*	*NE* within 50 % – 200 %?	*NT* within 50 % – 200 %?
N_{TOX}	**1**	78	85	82	76	95 %	97 %	Yes	Yes
	2	82	80	81					
	3	61	70	66					
N_{VIAB}	**1**	87	82	85	79				
	2	76	78	77					
	3	73	76	75					
N_{EFF}	**1**	71	79	75	75				
	2	75	63	69					
	3	84	78	81					

where

$$NE = \frac{N_{\mathrm{EFF}}}{N_{\mathrm{VIAB}}} \times 100 \qquad NT = \frac{N_{\mathrm{TOX}}}{N_{\mathrm{VIAB}}} \times 100$$

NOTE For abbreviations used in the formulae above, refer to the list in 3.2.

5.8 Procedure

5.8.1 Exposing the test and negative control dressings to test organisms[6]

5.8.1.1 In this procedure, the test dressing is tested in triplicate at the mandatory contact time of 24 h (T = 24 h) and the negative control dressing is tested in triplicate at both the initial time point (T = 0 h) and the mandatory contact time of 24 h (T = 24 h).

5.8.1.2 Place each sample of test dressing (three replicates per test organism) and negative control dressing (six replicates per test organism) in separate 55 mm Petri dishes with the wound contact side (if any) facing upwards.

5.8.1.3 Take *STOCK A* (5.6.3.5) and adjust to between $3{,}0 \times 10^6$ CFU/ml to $1{,}0 \times 10^7$ CFU/ml using SWF. This suspension is called *STOCK B*.

5.8.1.4 A count of the *STOCK B* prepared in 5.8.1.3 shall be performed to check the concentration used was in the correct range, using standard microbiological techniques on agar.

5.8.1.5 Each dressing sample shall be inoculated with a total volume corresponding to 80 % of total absorption capacity, which is the same as the calculated WV_C and WV_T (5.5.14). In this way, each dressing sample will be inoculated with $1{,}0 \times 10^6$ CFU to $5{,}0 \times 10^6$ CFU in total. The total amount to add is given by the following formula:

0,5 ml *STOCK B* + (*WV* − 0,5 ml) SWF

EXAMPLE

WV = 5 ml SWF. Therefore, amount required for each dressing sample:

0,5 ml *STOCK B* + 4,5 ml SWF

NOTE If a dressing with low absorption capacity is tested, where *WV* is less than 0,5 ml, then adjust *STOCK B* so the total amount of test organism added to the test sample will be within the range $1{,}5 \times 10^6$ CFU / sample to $5{,}0 \times 10^6$ CFU / sample (see D.11 in Annex D).

5.8.1.6 Prepare *INOC C* for the negative control dressing by mixing *STOCK B* and uninoculated SWF in proportions corresponding to:

INOC C = 0,5 ml *STOCK B* + (WV_C − 0,5 ml) SWF

5.8.1.7 Prepare *INOC T* for the test dressing by mixing *STOCK B* and uninoculated SWF in proportions corresponding to:

INOC T = 0,5 ml *STOCK B* + (WV_T − 0,5 ml) SWF

[6] For a graphical representation of (a) layout of samples in petri dish see Figure F.2, (b) Preparation of *STOCK B*, see Figure F.3 and (c) Preparation of *INOC C, INOC T* and addition to dressings, see Figure F.5 in Annex F.

5.8.1.8 An example of preparation of *INOC C* is given in Table 5. *INOC T* is prepared in the same manner as *INOC C* but with the volume adjusted for three samples.

Table 5 — Example preparation of *INOC C* for a hypothetical negative control dressing

WV_C	5,8 ml
STOCK B	0,5 ml
Uninoculated SWF = WV_C − *STOCK B*	5,8 ml − 0,5 ml = 5.3 ml
Volume required to inoculate all 6 samples = 6 × WV_C	6 × 5,8 ml = 34,8 ml (round up for nearest 10 ml)
Apply the following formula to calculate concentration of test organism per volume for *INOC C*: $C_C = \frac{C_s}{WV_c}$	

NOTE For abbreviations used in the formulae above, refer to the list in 3.2.

5.8.1.9 Distribute aliquots corresponding to WV_c of *INOC C* to the negative control dressings and WV_T of *INOC T* to the test dressings evenly across the surface of the dressing samples facing upwards in a manner that avoids run off of the inoculum. Transfer the dressing samples to fresh, unused Petri dishes, using sterile forceps, keeping the backing film, if present, facing downwards.

5.8.1.10 A count of the *INOC C* and *INOC T* prepared in 5.8.1.6 and 5.8.1.7 shall be performed to check the concentration used was in the correct range, using standard microbiological techniques on agar.

5.8.1.11 For the T = 0 h samples (three sample of the negative control dressings), proceed to step 5.8.2.

5.8.1.12 For the T = 24 h samples (three of the negative control dressings and three of the test dressings), place lids on Petri dishes containing these samples. Incubate in humid environment at 30 °C to 34 °C for 24 h ± 1 h, for example by placing the Petri dishes into plastic grip bags.

NOTE An acceptable alternative to grip bags can be a humidified incubator.

5.8.2 Recovery and enumeration of test organisms

5.8.2.1 At the end of the contact time (T = 0 h ± 5 min or T = 24 h ± 1 h), transfer each sample into a paddle blender bag containing the validated volume of neutralizer (5.7.4.2).

NOTE Additional contact times can be used as long as the viability of the negative control dressing has been validated for that time point.

5.8.2.2 Process in the paddle blender for the time and speed determined during neutralization validation (5.7.4.4). If an alternative recovery method to processing in a paddle blender is used, it shall be documented in the report and its use validated and justified. The alternative recovery method shall also be used in neutralization validation (5.7.4).

NOTE See D.16 in Annex D for information on the choice of paddle blender and possible alternative recovery methods.

5.8.2.3 Let the neutralizer react for the required time (validated in 5.7.4.4) at ambient temperature (15 °C to 25 °C). This suspension is considered as undiluted test suspension (N_0) i.e. 10^0.

5.8.2.4 For counting the microbial test suspensions from samples, prepare dilutions of the undiluted test suspension (N_0) using serial decimal dilutions in MRD to the appropriate dilution level for the samples. Ensure the undiluted test suspension (N_0) is suitability mixed (e.g. by hand squeezing the bag) before preparing the serial dilutions. The volume taken from the undiluted test suspension (N_0) to prepare the first serial decimal dilution is called *Vs*.

When preparing serial decimal dilutions, the usual volume taken (*Vs*) is 1 ml which is placed into 9 ml MRD. The recommended dilutions to be prepared are between 10^{-1} and 10^{-6}.

5.8.2.5 Each dilution is enumerated by duplicate pour plates. Transfer two 1,0 ml aliquots into separate 90 mm Petri dishes and add 20 ml to 25 ml melted agar (TSA for bacteria and SAA for yeast) cooled to 42 °C to 48 °C. Swirl gently to mix the contents of the dishes and allow to set at ambient temperature (15 °C to 25 °C). Spread plates may be used in place of pour plates, refer to 5.2.2.

The laboratory conducting the test can choose which are the most appropriate dilutions to plate out for each test. Where a test dressing is expected to be highly effective, only the N_0 and 10^{-1} dilution require plating. For the control dressing only plate out the higher dilutions, such as 10^{-4} to 10^{-6}.

5.8.2.6 Incubate the agar plates at 30 °C to 35 °C for bacteria and 20 °C to 25 °C for yeast, for 48 h to 72 h.

5.8.2.7 Count plates in the range 15 CFU to 300 CFU and record the result. For the undiluted test suspensions (N_0) record the CFU recovered even if the counts are < 15 CFU. If no CFU are recovered on any of the plates from the dilution series, record the number 0.

5.8.2.8 Calculate results and detection limit of the test according to 5.8.3 and 5.8.4.

5.8.3 Calculation and expression of results

5.8.3.1 Apply the following formula to calculate *N* resulting from each replicate of each test and control dressing:

$$N = \frac{P_1 + P_2}{2}$$

where

N is the average number of CFU on P_1 and P_2;

P_1 is the number of organisms on plate 1 of duplicate agar plates (CFU);

P_2 is the number of organisms on plate 2 of duplicate agar plates (CFU).

5.8.3.2 Apply the following formula to calculate the CFU/ml in the undiluted test suspension (N_0) (5.8.2.4):

$$C_V = N \times DF \times PF$$

where

C_V is are the viable counts per ml (CFU/ml);

N is the average number of CFU on P_1 and P_2;

DF is the dilution factor (1 for neat, 10 for 10^{-1}, 100 for 10^{-2}, 1 000 for 10^{-3}, etc.);

PF is the plate factor (1 for 1 ml pour plate, 10 for 0,1 ml spread plate, 2 for 0,5 ml spread plate, etc.).

5.8.3.3 Apply the following formula to calculate the viable counts per replicate at each time point:

$$C_T = C_V \times V_T$$

where

6 are the viable counts per dressing sample (C_0, C_{24}, or T_{24}) (CFU/ml);

C_V are the viable counts per ml (CFU/ml);

V_T is the test volume = neutralizer volume (V_N) + working volume (WV_T or WV_c) (ml).

5.8.3.4 An example of the calculation of the negative control dressing at a contact time 0 h (T = 0 h) and 24 h (T = 24 h) and a test dressing at a contact time 24 h (T = 24 h) is given in Table 6:

Table 6 — Example of calculations for a theoretical negative control and test dressing (using 1 ml pour plates)

Negative control dressing example calculations (C_0)															
Sample	**Time** (h)	**Rep.**	P_1 (CFU)	P_2 (CFU)	PF	N (CFU)	DF (ml)	C_v (CFU/ml)	V_N (ml)	WV_C (ml)	V_T (ml)	C_0 (CFU)	Log C_0	**Average** Log C_0	C_0 **within** range?
Negative control dressing	0	1	132	124	1	128	1 000	$1{,}28 \times 10^5$	20	0,6	20,6	$2{,}64 \cdot \times 10^6$	6,42		
		2	115	123	1	119	1 000	$1{,}19 \times 10^5$	20	0,6	20,6	$2{,}45 \cdot \times 10^6$	6,39	6,41	Yes
		3	123	127	1	125	1 000	$1{,}25 \times 10^5$	20	0,6	20,6	$2{,}58 \cdot \times 10^6$	6,40		
Negative control dressing example calculations (C_{24})															
Sample	**Time** (h)	**Rep.**	P_1 (CFU)	P_2 (CFU)	PF	N (CFU)	DF (ml)	C_v (CFU/ml)	V_N (ml)	WV_C (ml)	V_T (ml)	C_{24} (CFU)	Log C_0	**Average** Log C_{24}	**Spread** ≤ 1,0?
Negative control dressing	24	1	98	92	1	95	100 000	$9{,}50 \times 10^6$	20	0,6	20,6	$1{,}96 \cdot \times 10^8$	8,29		
		2	236	250	1	243	100 000	$2{,}43 \times 10^7$	20	0,6	20,6	$5{,}01 \cdot \times 10^8$	8,70	8,40	Yes
		3	70	86	1	78	100 000	$7{,}80 \times 10^6$	20	0,6	20,6	$1{,}61 \cdot \times 10^8$	8,21		
Test dressing example calculations (T_{24}) and antimicrobial activity (A)															
Sample	**Time** (h)	**Rep.**	P_1 (CFU)	P_2 (CFU)	PF	N (CFU)	DF (ml)	C_v (CFU/ml)	V_N (ml)	WV_T (ml)	V_T (ml)	T_{24} (CFU)	Log T_{24}	**Average** Log T_{24}	A
Test dressing	24	1	0	0	1	0	1	$< 1 \times 10^1$	20	0,7	20,7	$< 2{,}07 \times \cdot 10^1$	< 1,32		
		2	0	0	1	0	1	$< 1 \times 10^1$	20	0,7	20,7	$< 2{,}07 \times \cdot 10^1$	< 1,32	< 1,32	≥ 5,1
		3	0	0	1	0	1	$< 1 \times 10^1$	20	0,7	20,7	$< 2{,}07 \cdot \times 10^1$	< 1,32		

where

$$N = \frac{P_1 + P_2}{2} \times PF \qquad C_V = N \times DF \qquad V_T = V_N + WV_C$$

$$C_0 = C_V \times V_T \qquad C_{24} = C_V \times V_T \qquad T_{24} = C_V \times V_T$$

NOTE For abbreviations used in the formulae above, refer to the list in 3.2.

5.8.3.5 Calculate the antimicrobial activity (A) according to the following formula:

$$A = \text{Log } C_0 - \text{Log T}_{24}$$

where

A is the antimicrobial activity;

Log C_0 is the average log value for the number of organisms obtained from three negative control dressing samples immediately after inoculation;

Log T_{24} is the average log value for the number of organisms obtained from three test dressing samples after incubation for 24 h;

5.8.3.6 An example of calculating A is included in Table 6.

5.8.3.7 Report the antimicrobial activity (A) to 1 decimal place.

5.8.3.8 If the counts for P_1 and P_2 are zero (0) on the undiluted test suspension (N_0) plates, then the detection limit value (LOD calculated in 5.8.4.1) shall be used for the calculation in 5.8.3.5 and reported as "< LOD CFU / sample" or "< LOD Log CFU / sample". Subsequent calculation of the antimicrobial activity value (A) shall then be reported as a "≥" value as shown in the example calculation in Table 6.

5.8.4 Calculation of the detection limit of the test

5.8.4.1 The detection limit (LOD) of a test is the lowest theoretical detectable number of microorganisms that can possibly be recovered from the specified test. This value is dependent on the total volume of the test V_T (which in turn is the sum of WV and V_N), the volume sampled from the paddle blender bag (V_S), the dilution factor used and the plate factor. The LOD is calculated as follows:

$$LOD = \frac{V_T \times V_S}{DF} \times PF$$

where

LOD is the limit of detection;

V_T is the test volume = neutralizer volume (V_N) + working volume (WV_T or WV_C) (ml);

V_S is the volume sampled from the paddle blender bag when preparing serial decimal dilutions in 5.8.2.4 (ml);

DF is the dilution factor (1 for neat, 10 for 10^{-1}, 100 for 10^{-2}, 1 000 for 10^{-3}, etc.);

PF is the plate factor (1 for 1 ml pour plate, 10 for 0,1 ml spread plate, 2 for 0,5 ml spread plate, etc.).

5.8.4.2 An example of an *LOD* calculation is given in Table 7. When counts on the plates are 0 the results are recorded as:< 10 CFU/sample or < 1,0 Log CFU/sample.

Table 7 — Example of calculation of *LOD* for an example dressing using pour plates

Working volume (WV_T)	0,6 ml
Neutralizer volume in the paddle blender bag (V_N)	20 ml
Volume sampled from paddle blender bag (V_S)	1 ml
Dilution factor (*DF*)	1
Plate factor (*PF*)	1
where $$LOD = \frac{V_T \times V_S}{DF} \times PF$$	
LOD = 20,6 CFU or 1,3 log CFU	

NOTE For abbreviations used in the formulae above, refer to the list in 3.2.

5.8.5 Judgement of test validity

5.8.5.1 Conditions a), b) and c) below shall be met for the test to be judged as valid. When the test is judged to invalid the test may be repeated.

a) The mean negative control dressing count at the initial contact time (T = 0 h) shall be within the range $1,0 \times 10^6$ CFU / sample to $5,0 \times 10^6$ CFU / sample.

b) The spread for the three negative control dressings after incubation at contact time 24 h (T = 24 h) shall be ≤ 1,0 log.

c) The conditions of neutralizer validation in 5.7 are met.

5.8.5.2 The method of recovery from the dressings shall be effective and is only valid if ≥ 50 % and ≤ 200 % of the theoretical number of microorganisms added to the T = 0 h negative control dressing can be recovered. This may be calculated either from the data obtained by comparing the inoculum count to the T = 0 h negative control dressing results or by validating the method of recovery as a separate study.

NOTE The limit of ≥ 50 % and ≤ 200 % has been chosen to mirror that used in neutralization validation (5.7).

5.9 Test report

Test reports shall include the following information as a minimum:

5.9.1 Reference to this document (including year of publication).

5.9.2 All details necessary for complete identification of the sample, including a description and unambiguous identification of the sample tested. The following information should be available for commercial dressings and included in the report: type of dressing/dressing name, manufacturer or supplier of the dressing, batch number, expiry date, catalogue number, product code or SKU (stock keeping unit).

5.9.3 Test parameters including:

— date of testing;

— test organism strains including culture reference, or sufficient descriptive information of the isolate if an additional test organism is used and there is no culture collection reference (e.g. *Staphylococcus aureus* clinical isolate from leg ulcer);

— details of the neutralizer including neutralizer composition, volume used and standing time required for effective neutralization;

— time and speed used for processing samples in the paddle blender;

— concentration of *STOCK N*, *STOCK B*, *INOC C* and *INOC T* for each test organism;

— any deviations from the method, with justification, including additional contact times, variations of test organisms or sub-culturing and plating methodology or recovery method if changed from paddle blender;

— any unusual features observed during the test.

5.9.4 Test results including:

— calculated viable counts per test dressing and negative control dressing;

— antimicrobial activity value (A);

— judgement of test validity;

— neutralization validation data for each test strain including the calculated neutralizer toxicity (N_{TOX}), neutralizer efficacy (N_{EFF}) and test organism viability (N_{VIAB}).

Annex A
(informative)

Referenced test organism strains in other national collections

Pseudomonas aeruginosa	ATCC 9027
	CIP 82.118
	DSM 1128
	NBRC 13275
	CCUG 22801
	NCIMB 8626
	NCTC 12924
Staphylococcus aureus	ATCC 6538
	CIP 4.83
	DSM 799
	NBRC 13276
	CCUG 10778
	NCIMB 9518
	NCTC 10788
Candida albicans	ATCC 10231
	CIP 48.72
	DSM 1386
	NBRC 1594
	CCUG 19915
	NCPF 3179

Key

ATCC	American Type Culture Collection (USA)
CIP	Pasteur Institute Collection (France)
DSM	German Collection of microorganisms and Cell Cultures (Germany)
NBRC	NITE Biological Resource Centre (Japan)
CCUG	Culture Collection, University of Gothenburg (Sweden)
NCIMB	National Collection of Industrial Bacteria (UK)
NCTC	National Collection of Type Cultures (UK)
NCPF	National Collection of Pathogenic Fungi (UK)

Annex B
(informative)

Validation of neutralization

B.1 Principle

The effectiveness of antimicrobial agents incorporated into wound dressings is measured by their ability to prevent growth or kill microorganisms within a specified contact time. Consequently, accurate determination of antimicrobial activity requires complete and immediate neutralization of the antimicrobial agent. Inefficient or incomplete neutralization will permit killing or inactivation of microorganisms by the antimicrobial agent to continue beyond the experimental exposure time, resulting in an overestimation of antimicrobial activity. A neutralizer is therefore required for each antimicrobial dressing to inactivate or quench the microbicidal properties of the antimicrobial agent.

Validation of neutralization consists of three experimental tests: neutralizer toxicity, test organism viability and neutralization efficacy for each test strain.

Neutralizing agents may also be incorporated into agar to increase the neutralizing capacity. Agar containing neutralizing agents may be used for the enumeration of microbial test suspensions providing it has been evaluated following the same principles as described in 5.7; that neutralization effectiveness has been validated and it does not have an inhibitory effect on organism viability.

The working group acknowledge that there are variations in published methodologies for conducting validation of the neutralizing solution. The basic principles of checking for toxicity and effectiveness of neutralization are common across all methodologies.

B.2 Neutralizer selection

Refer to Annex C for examples of suitable neutralizers for a range of antimicrobial agents.

The neutralizer selected should, where possible, minimize the volume required to allow the use of 1 ml agar pour plates to be used from the neat dilution, therefore maximizing the sensitivity of the test.

Annex C
(informative)

Neutralizers

Table C.1 — Examples of active agents and possible neutralizers

Antimicrobial agent	Neutralizing agent
Silver	Sodium thiosulphate / sodium thioglycolate
Quaternary ammonium agents (Benzalkonium chloride)	Lecithin/polysorbate
Chlorhexidine	Polysorbate 80 / egg lecithin
Polyhexamethylene biguanide (PHMB)	Polysorbate 80 / asolectin
Honey	Lecithin
Iodine	Sodium thiosulphate and lecithin

NOTE 1 The above list is not exhaustive and other neutralizers can be tried. See EN 1040 [4] or EN 13727 [5] for further examples and composition.

NOTE 2 Dey-Engley contains a wide range of neutralizers and was found to be a good first choice when choosing a neutralizer by the working group.

Annex D
(informative)

Rationale

D.1 General

This annex is intended to explain how and why certain decisions have been made concerning this document by the working group.

D.2 Title

The use of the term 'antimicrobial' has been specifically chosen to enable this document to include methods other than microbicidal when available.

D.3 Test organism strains

This document presently includes tests for two bacteria and a yeast. Future editions will consider the inclusion of mold and additional test organisms. The organisms used in this document (5.3.1) have been selected based on their use in other antimicrobial standards and are good examples of their species.

Specifically:

— *Staphylococcus aureus* is the most commonly isolated organisms from acute and chronic wounds. The strain ATCC 6538 is chosen since it is a clinical isolate and is it also specified in ISO 22196 [6] and EN ISO 20743 [7] (antimicrobial testing of plastics and textiles, respectively).

— *Pseudomonas aeruginosa* is commonly isolated from both acute and chronic wounds. The strain ATCC 9027 is chosen since it is a clinical isolate and it is specified in disinfectant standards EN 1040 [4], EN 1276 [8] and EN 13727 [6].

— *Candida albicans* is part of the normal microflora (and therefore an opportunistic pathogen) of various ecological niches on the human body including skin. It acts as an opportunistic pathogen in immunocompromised patients or those on broad spectrum antibiotics and is therefore a key infection-causing organism across many different wound types. It is also a commonly isolated yeast from chronic wounds. The strain ATCC 10231 is chosen since it is a clinical isolate and is specified in disinfectant standards EN 13624 [9], EN 14562 [10] and EN 1657 [11].

Users of this document may use additional test organism species in addition to those specified in 5.3.1 provided appropriate neutralizer validation has been undertaken to determine their suitability and there is a full explanation given in the test report.

Whilst the working group understand there is interest in effectiveness against antibiotic resistant organisms, there is no requirement in this document to use such organisms.

D.4 Preparation of test organism suspensions

It is usual that stock cultures are stored frozen, usually in glycerol at −70 ˚C as per EN 12353. However, other stocks may be available depending on the laboratory's procedures (e.g. ready prepared cultures in vials or loops) which are also acceptable.

Preparing inoculum from surface grown cells on agar was included in this document (5.6) as such cells are believed to be more resistant due to different surface characteristic (examples published in Ljungh *et al* [12]).

Harvesting from overnight cultures using centrifugation is used by some laboratories and is intended to provide cells free from extracellular matrix which are in a more uniform growth phase (stationary) compared to cells grown up in broth to a particular Optical Density (OD) on the day of testing. This was included as part of test organism preparation during the initial drafts of this document but was removed as the working group acknowledged that not all laboratories have an appropriate centrifuge and it could provide a barrier to its use.

D.5 Cutting dressings

To obtain appropriate samples for the tests in this document it is frequently necessary to cut the dressings under aseptic conditions. Depending on the dressing design and construction, this action may disrupt the dressing matrix and release antimicrobial agents which would not necessarily be released from an intact dressing, potentially enhancing the dressings performance during the test, which might not be reflected in the performance of an intact dressing.

The working group acknowledge that this is potentially problematical, but that at the time of writing, it reflects the state of the art in dressings testing.

In the future it may be possible to deal with this issue by including a test for an intact dressing using a wound model such as that described by Walker *et al* [13].

The working group are interested in any proposals for test methods for intact dressings for future revisions.

D.6 Positive control

The working group acknowledge that this document does not include a positive control as a suitable positive control for this test method could not be identified. However, the working group is interested in any proposals for a suitable positive control for inclusion in a future revision.

D.7 Performance requirements

The method is designed to show antimicrobial activity *in vitro*. Whilst the method is not correlated with clinical activity, there is an expectation that a product demonstrating microbicidal activity *in vitro* is able to exert a clinical effect *in vivo* under suitable conditions. Peterson *et al* [14] noted that the selection of a 3 log reduction for the minimum bactericidal concentration (i.e. the lowest concentration which results in a 99,9 % reduction in the initial inoculum) is an arbitrary endpoint but is well accepted and has been used as the minimum reference value for many years.

The requirement for a microbistatic dressing to meet $A \geq 0$ is based on the following rationale:

If $A \geq 0$ then the growth on the test dressing is the same as or lower than that seen on the negative control dressing. If A is < 0, then there has been growth with the test dressing. A value of < 3 shows that the dressing has not achieved the 3 log reduction necessary to meet the requirements for a microbicidal effect.

D.8 Media

Wound exudate contains salts and proteins, which are known to interfere with some antimicrobial substances used for dressings. Therefore, this test procedure uses a simulated wound fluid (SWF), which also contains salts and proteins, for suspension of the test organisms when exposed to products containing antimicrobial substances to mimic wound fluid conditions.

Fetal calf serum (FCS) was chosen for use in the SWF by the working group as a source of salts and proteins, rather than alternatives such as serum albumin or sheep erythrocytes. The working group found less variability with FCS during inter-laboratory trials conducted during development of this document. It is for this reason that substitution of FCS with an alternative reagent in the SWF is not recommended (5.3.2.1).

D.9 Incubation temperatures

The incubation temperatures for the tests are based on temperatures found in wounds as described in McGuiness *et al* [15].

The incubation temperatures for the organism recovery of 30 °C to 35 °C for bacteria and 20 °C to 25 °C for yeasts are based on the requirements in the European and United States Pharmacopoeias.

D.10 Dressing classification

The working group note that there is no definition or assessment in this document of whether a dressing contains a 'releasing' or 'non-releasing' active substance and future editions may reconsider whether this would be a useful classification.

D.11 Saturation volume

The working group acknowledge that comparing different types of dressings (for example, comparing alginates with foams) may be problematic. The saturation volume and adjustment of the test organism suspension so that each sample contains the same challenge (CFU) is intended to address this issue.

The working group have not tested any non-absorbent or low-absorbent dressings during development of this document and therefore cannot recommend a lower volume than 0,5 ml to use for *STOCK B* (5.8.1.5). This may be considered for future versions of this document.

It is known that the density of water changes depending on the temperature at which it is being used and this is usually accounted for when converting weight to volume of solutions. However, due to the potential variation in MRD and FCS from different suppliers, the working group could not pre-determine the density of these solutions. Therefore, the working group considered it to be sufficiently accurate for the test method in this document to assume equivalency between weight and volume (i.e. 1 g = 1 ml) when calculating the saturation volume (see 5.5).

D.12 Procedure

The procedure used in this document (known as "Direct Contact" method) is based upon the method referenced in Gallant-Behn *et al* [16]. It is suitable for most dressing types, but the recovery for super absorbent dressings may be limited.

D.13 Contact times

Testing the dressing at time zero (T = 0 h) was not considered necessary as the antimicrobial activity is calculated from the time 0 h (T = 0 h) negative control dressing in this document. However, this may be incorporated into the test if desired.

D.14 Pre-conditioning and repeat challenge testing

The working group notes that some regulatory authorities may require incorporation of other test parameters not covered in this document. The most notable are (a) pre-conditioning the test dressings with serum prior to inoculating with the test organism and (b) repeat challenge testing where additional inoculation(s) with test organisms are performed after the mandatory 24 h contact time (T = 24 h). The working group acknowledges that these parameters may provide more robust challenges to the test dressings. As these parameters were not covered during the inter-laboratory comparisons, they were unable to be incorporated into this document, however they may be considered for future editions.

D.15 Humidity

No tolerances for humidity have been stated in this document as the working group did not examine the impact of different humidity ranges on the test. The most common method used in the trials was placing petri dishes into grip bags to create a humid environment. There is no requirement to measure/record humidity in the test however labs may wish to do this and provide the data in the test report.

D.16 Recovery of test organisms from dressings

Paddle blenders are used in this document as it is the experience of the Working Group that it is the most appropriate method for recovery of organisms from a wide range of wound dressings and especially for foam dressings. However, the paddle blender processing time and speed will vary for different wound dressings. The bioburden recovery efficiency guidance documented in EN ISO 11737-1 [17] may be used to determine the most suitable paddle blender processing time and speed that ensure sufficient recovery of organisms from samples. This work would be carried out prior to conducting neutralization validation (5.7) to reduce the chance of invalid results and having to perform repeat experiments.

There is no separate calculation of Recovery Efficiency (as defined within EN ISO 11737-1 [17]) in this document. This is because the Neutralizer Effectiveness calculations (5.7.5.4) ensures that there is sufficient and effective recovery of organisms based on a combination of the neutralizer used and the paddle blender processing parameters.

If an alternative recovery method to a paddle blender is deemed to be more suitable for the dressing to be tested then it can be used provided this is justified and noted in the test report (5.7.4.4). Alternative recovery methods that the Working Group are aware of includes sonicating for 5 min or vortexing for 1 min to 2 min with sterile glass beads.

Annex E
(informative)

Replicates

It was not feasible within the working group to do a sufficient number of inter-laboratory comparisons on a suitable number of dressing types to ascertain uncertainty, precision, and repeatability of the method in this document. However, the working group has reviewed a number of similar antimicrobial standards and note that many of them have standardized on three replicates.

Some of the documents reviewed are detailed below:

— EN ISO 20743 [7] (textile antimicrobial) requires three replicates. No limit is given for the acceptable variation between test samples but for controls, the difference in common log values should be < 1 log.

— ISO 22196 [6] (plastic antimicrobial) requires a minimum of three replicates. Variation between controls (the range) at the start shall be ≤ 0,2, when calculated using the following formula:

$$\frac{\log(\max) - \log(\min)}{\log(\text{mean})} \leq 0,2$$

— ASTM E2149 [18] requires one replicate, and the control and inoculum should be within 15 %.

— AATCC TM100 [19] has no information on the number of replicates required.

— ISO 21702 [20] (hard surfaces) and ISO 18184 [21] (textiles) for antiviral testing use three replicates. One of the Project Group laboratories has extensive experience of using these standards and confirm that the use of three replicates is sufficient.

— The disinfectant standards examined do not state a minimum number of replicates. EN 17126 [22] and EN 13727 [6] state that for a precision of ±1 log then four replicates at best and six replicates at worst.

Overall, the majority of standards use three replicates, and these are widely accepted as being practical and the minimum required for a microbiological test.

During interlaboratory testing for the test method in this document, it was found that when using triplicate experiments, more than 95 % of the negative control dressings at T = 0 h had a range of ≤ 0,2 for the three replicates and 93 % of the antimicrobial activity log reduction calculations had a standard deviation of ≤ 0,2. These data were reviewed and accepted by the working group as justification for using three replicates when performing the test method described in this document.

Laboratories performing this test should be aware of the potential for greater variability than was found in the interlaboratory experiments described above when testing different dressings. Therefore, in such cases the laboratory may increase the number of replicates tested.

Annex F
(informative)

Test method illustrations

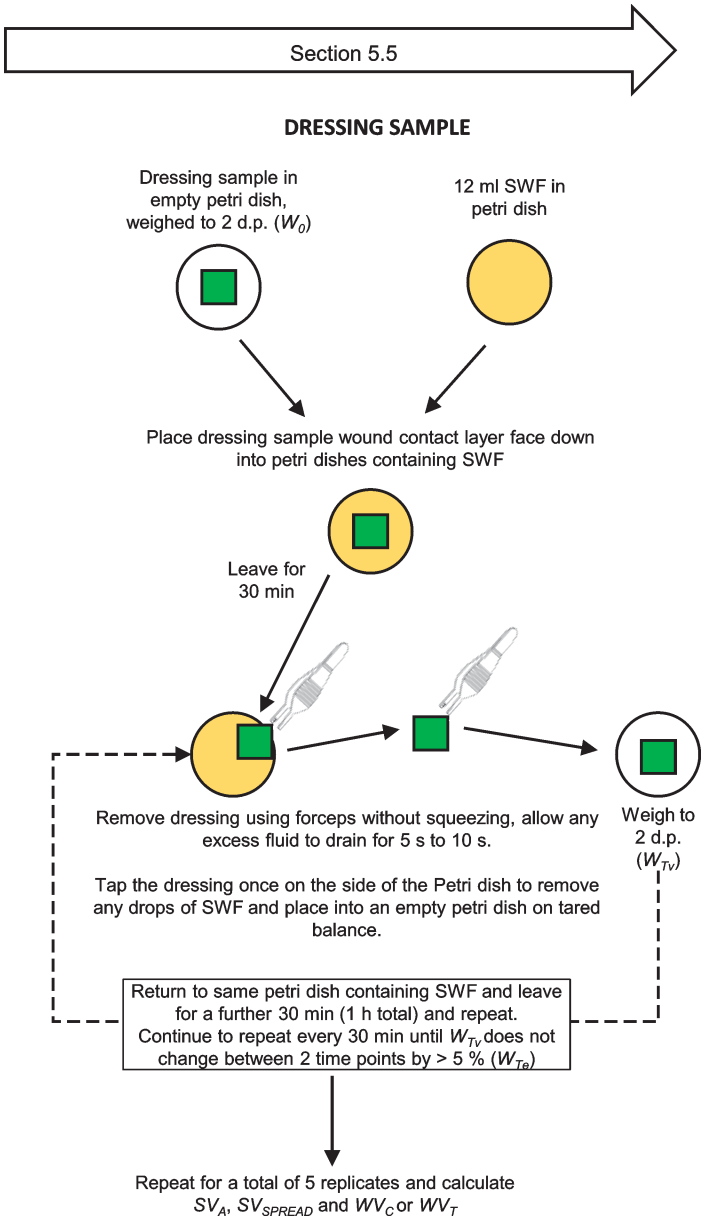
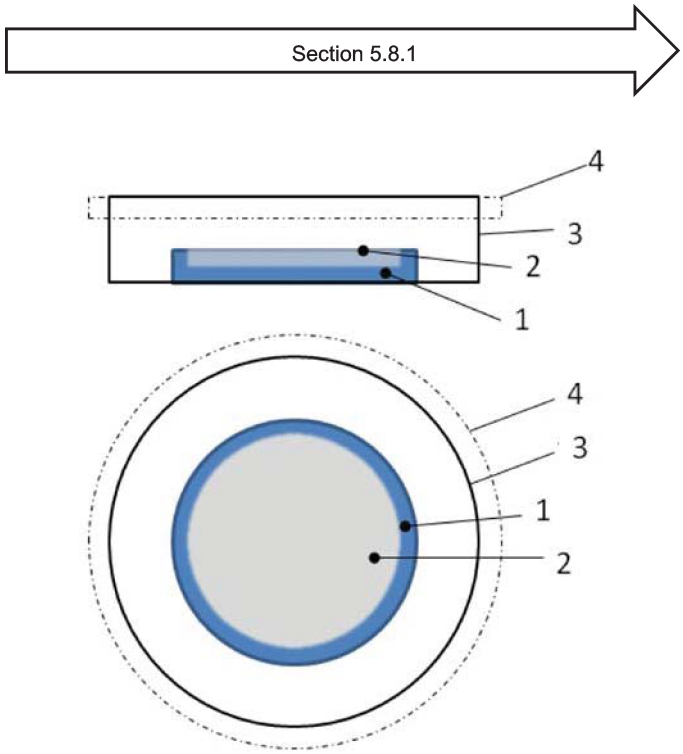


Figure F.1 — Saturation volume and working volume



Key

1 sample
2 inoculum, including working vol (3.1.12)
3 Petri dish
4 lid of a Petri dish

Figure F.2 — Layout of samples in petri dish

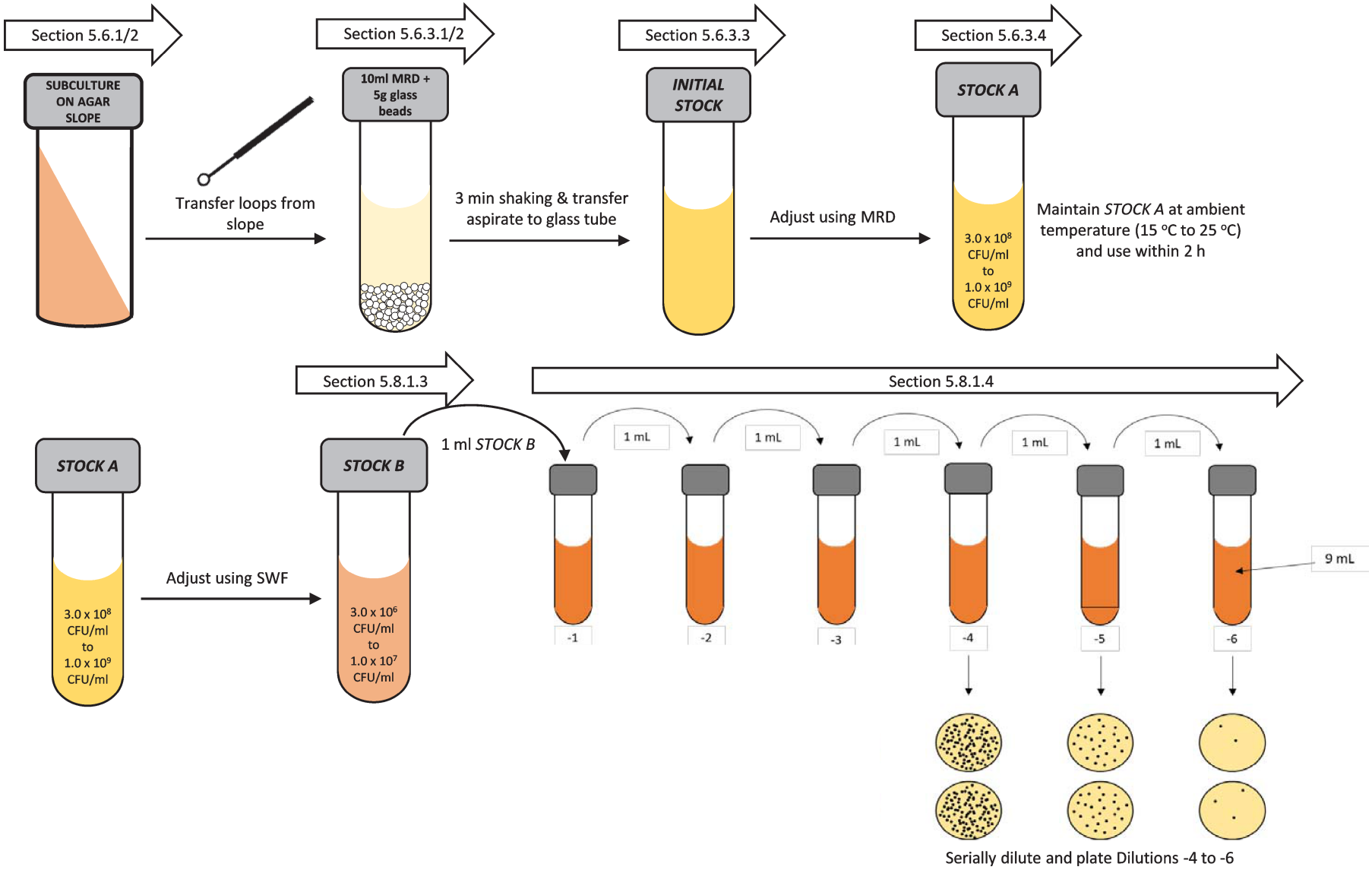
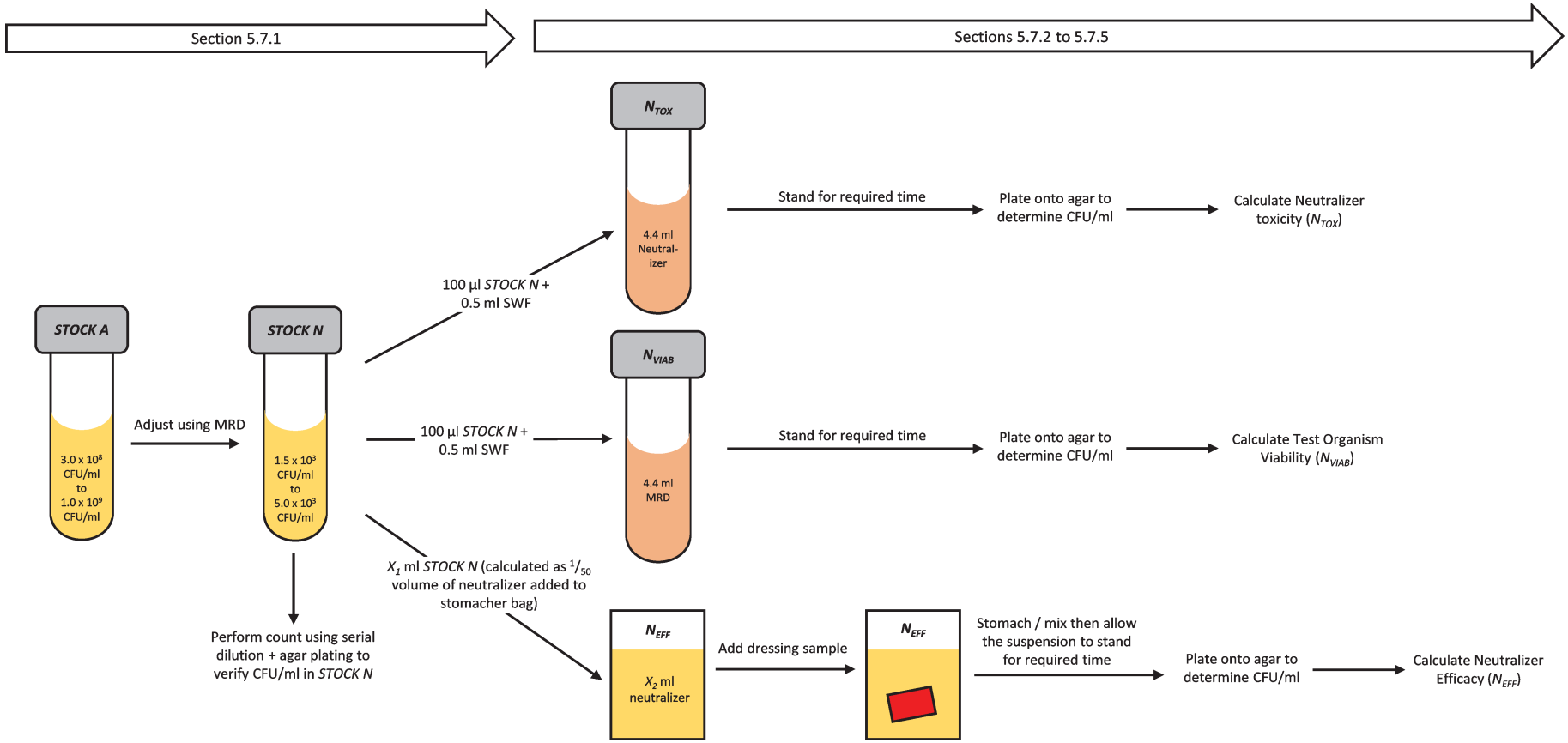


Figure F.3 — Production of *STOCK A* and *STOCK B*

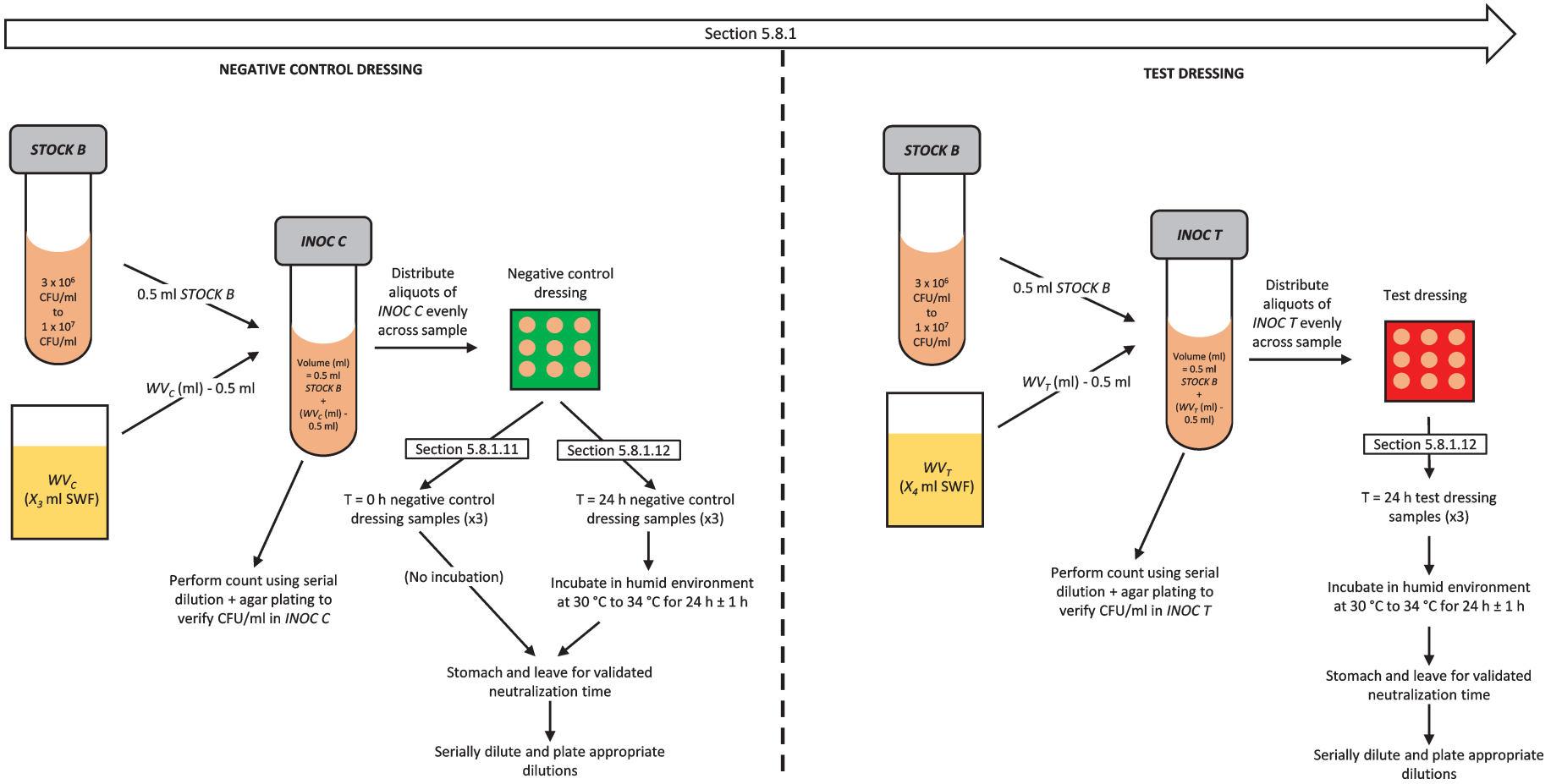


Key

X_1 volume (ml) of *STOCK N* from 5.7.4.3

X_2 volume (ml) of neutralizer from 5.7.4.2

Figure F.4 — Neutralization validation



Key

X_3 calculated volume of SWF from 5.8.1.6

X_4 calculated volume of SWF from 5.8.1.7

Figure F.5 — Preparation of *INOC C*, *INOC T* and addition to negative control and test dressings

Annex G
(informative)

Example Test Report Tables

These are example tables only that can be included in the test report. Refer to 5.9 for full details required in the report.

Table G.1 — Example for a test report results table (blank)

Test dressing _____				
Negative control dressing _____				
Exposure time _____				
Test organism	**Dressing**	**Time** 0 h (T = 0 h) **mean Log counts per sample (C_0)**	**Time** 24 h (T = 24 h) **mean Log counts per sample (C_{24} and T_{24})**	**Antimicrobial Activity** (A)
S. aureus	Negative control			
	Test	—		
P. aeruginosa	Negative control			
	Test	—		
C. albicans	Negative control			
	Test	—		
Performance: Microbiocidal/Microbistatic/Non-antimicrobial (delete as appropriate)				

Table G.2 — Example of a completed Microbicidal test report results table

Test dressing Silver foam				
Negative control dressing Silver-free foam				
Exposure time 24 h				
Test organism	**Dressing**	**Time** 0 h (T = 0 h) **mean Log counts per sample (C_0)**	**Time** 24 h (T = 24 h) **mean Log counts per sample (C_{24} and T_{24})**	**Antimicrobial Activity** (A)
S. aureus	Negative control	6,3	6,7	−0,4
	Test	—	2,1	4,2
P. aeruginosa	Negative control	6,4	7,0	−0,6
	Test	—	1,2	4,9
C. albicans	Negative control	6,1	6,2	−0,1
	Test	—	< 1,6	> 4,5
Performance: Microbicidal				

Table G.3 — Example of a completed Microbicidal test report results table

Test dressing Silver foam				
Negative control dressing Silver-free foam				
Exposure time 24 h				
Test organism	**Dressing**	**Time** 0 h (T = 0 h) **mean Log counts per sample (C_0)**	**Time** 24 h (T = 24 h) **mean Log counts per sample (C_{24} and T_{24})**	**Antimicrobial Activity** (A)
S. aureus	Negative control	6,3	6,7	−0,4
	Test	—	4,1	2,2
P. aeruginosa	Negative control	6,4	7,0	−0,6
	Test	—	5,3	0,9
C. albicans	Negative control	6,1	6,2	−0,1
	Test	—	2,6	4,5
Performance: Microbistatic				

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[3] EN ISO 11133, *Microbiology of food, animal feed and water - Preparation, production, storage and performance testing of culture media (ISO 11133)*

[4] EN 1040, *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)*

[5] EN 13727, *Chemical disinfectants and antiseptics. Quantitative suspension test for the evaluation of bactericidal activity in the medical area. Test method and requirements (phase 2, step 1)*

[6] ISO 22196, *Measurement of antibacterial activity on plastics and other non-porous surfaces*

[7] EN ISO 20743, *Textiles - Determination of antibacterial activity of textile products (ISO 20743)*

[8] EN 1276, *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)*

[9] EN 13624, *Chemical disinfectants and antiseptics - Quantitative suspension test for the evaluation of fungicidal or yeasticidal activity in the medical area - Test method and requirements (phase 2, step 1)*

[10] EN 14562, *Chemical disinfectants and antiseptics - Quantitative carrier test for the evaluation of fungicidal or yeasticidal activity for instruments used in the medical area - Test method and requirements (phase 2, step 2)*

[11] EN 1657, *Chemical disinfectants and antiseptics - Quantitative suspension test for the evaluation of fungicidal or yeasticidal activity of chemical disinfectants and antiseptics used in the veterinary area - Test method and requirements (phase 2, step 1)*

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[18] ASTM E2149, *Determining the Antimicrobial Activity of Immobilized Antimicrobial Agents Under Dynamic Contact Conditions*

[19] AATCC TM100, *Test Method for Antibacterial Finishes on Textile Materials: Assessment of*

[20] ISO 21702, *Measurement of antiviral activity on plastics and other non-porous surfaces*

[21] ISO 18184, *Textiles - Determination of antiviral activity of textile products*

[22] EN 17126, *Chemical disinfectants and antiseptics - Quantitative suspension test for the evaluation of sporicidal activity of chemical disinfectants in the medical area - Test method and requirements (phase 2, step 1)*