Preparation and Validation of Bioindicator Enclosures for Use in Industrial Laundry Disinfection Process Test

1. Scope

This method is used to validate new bioindicator enclosures or membranes to hold contaminated textile samples in the laundry disinfection process test, evidence based on Owen et al, 2024. The enclosures should be permeable to laundry chemistries, in order that the chemicals can come into contact with the contaminated textile samples, but prevent bacteria from passing out and being lost in the wash process.

The test enclosure must pass validation tests 1 and 2 in order to be used for validation of microbiological disinfection of laundry processes. The full procedure for preparing bioindicators for use in *Test One: Microbicidal Activity of Wash Process – Validation using Bioindicators* (Annex I, Sections 2-4) is outlined below, see supplementary material Section 5 for the preparation of in house bioindicators.

2. Test 1: Permeability of Enclosures to Laundry Chemicals

2.1. Principle

The test involves determining the permeability of the test enclosure by assessing the antimicrobial activity of a range of laundry chemistries against cotton samples contaminated with *Enterococcus faecium* sealed within the test enclosure, compared to loose cotton samples. Cotton textile samples are contaminated with a known quantity of *E. faecium.* Loose cotton samples are immersed in the test chemical in a solution as a control to calculate the antimicrobial activity of the chemical, while cotton samples enclosed in the test enclosure are laundered with an equivalent concentration of the test chemical. The $\log_{10}$ reduction per textile sample ($1cm^2$) of viable *E. faecium* is determined and compared between the treatments. If the $\log_{10}$ reduction is equivalent between the samples, this suggests that the chemical is passing through the enclosure and acting on the microorganism, and the membrane is suitable for preparing bioindicators used in the laundry disinfection process test. If the $\log_{10}$ reduction on the cotton sample within the tested enclosure is lower compared to that of the loose cotton sample, this indicates the membrane tested is not suitable for the laundry validation methodology.

2.2. Materials

2.2.1. Microorganisms

Enterococcus faecium NCIMB 2699 shall be cultured at 37°C for 48 hours on nutrient agar.

2.2.2. Nutrient agar

Pre-poured (commercial) or prepared in house (as per the manufacturers) into 90mm sterile Petri dishes.

2.2.3. Test Chemicals

Any chemicals with a disinfection purpose to be used within the wash process must be tested for validation of the test enclosure.

The concentration used in the test should produce a 2-3 $\log_{10}$ reduction of *E. faecium* on loose cotton textile samples; this concentration can be determined by performing the test with a range of concentrations of the test chemical prior to conducting the validation test. All additional chemicals must be neutralised using a neutraliser validated according to Test 3 prior to performing Tests 1-2. Examples of potentially suitable neutralising formulations for disinfectant chemicals are provided in BS EN ISO 13727:2012+A2:2015 Annex B.

In addition, the following chemicals must be used as detergent and disinfectant controls:

- Sodium dodecyl sulfate (SDS), 0.06% w/v (detergent).
- Peracetic acid, 0.0032% or sodium hypochlorite (chlorine) bleach, 0.016% (disinfectant).

2.2.4. Diluent

Phosphate buffered saline (PBS) solution, prepared with distilled water and sterilised by autoclaving.

2.2.5. Textile

Woven 100% cotton, 140 g/m^2, yarn count 20/20, thread count 120. Cut into 1 cm^2 samples and sterilised by autoclaving.

2.2.6. Washing machine ballast load

50/50 polyester/cotton sheets; AATCC Ballast Type 3 specification or similar e.g. BS EN ISO 6330:2021 ballast type II, totalling one-third the weight capacity of the washing machine. Ballast loads should be laundered at 90°C between wash cycles and sterilised by autoclaving prior to use.

2.2.7. Pillowcase

50/50 polyester/cotton standard pillowcase, sterilised by autoclaving prior to use.

2.2.8. Test Enclosure

The test enclosure should be sterilised prior to use. The enclosure should be sealed around test fabric specimens by suitable means (e.g. by heat sealing).

2.2.9. Washing Machine

A washer extractor equipped with a cold wash cycle consisting of the following parameters:

Stage 1, Pre-Wash: 4 minutes.

Stage 2, Main Wash: 10 minutes.

Stage 3, Rinse 1: 2 minutes.

Stage 4, Rinse 2: 2 minutes.

Stage 5, Rinse 3: 2 minutes.

Stage 6, Extract: 5 minutes, extract spin.

Additional tests may also be conducted with a 4-minute pre-wash at 40°C and 10-minute main wash cycle up to 60°C, for example where membrane permeability is limited at ambient temperature.

The water volumes used in the main wash cycle should be known. The washing machine shall be flushed using a 90°C wash cycle between test runs.

2.2.10. Membrane filters and filtration unit

Sterile cellulose acetate, 0.45 µm and vacuum filtration unit.

2.3. **Test Method**

2.3.1. Inoculum preparation

a) Take a loopful of *E. faecium* NCIMB 2699 using a sterile loop and streak on nutrient agar. Incubate the streak plate for 24 hours at 37°C.
b) Aseptically transfer loopfuls of *E. faecium* colonies to sterile PBS to produce a suspension containing 10^8 colony forming units (CFU)/ml *E. faecium.* The inoculum concentration may be verified according to any suitable means. For example, measurement of optical density at 600 nm using a spectrophotometer may be used to verify inoculum concentrations by comparing optical density to a standard curve of optical density versus CFU/ml produced using known inoculum sizes. A calibration curve should be produced for each laboratory and for each microorganism to be tested (BS EN 13727:2015).

c) Prepare two separate suspensions per test (n=2). Use the suspensions immediately after preparation.
d) Verify the inoculum load by serially diluting in PBS and spread plating on nutrient agar. Incubate at 37°C for 48 hours before counting the number of colonies. Calculate the $\log_{10}$ CFU/ml. The inoculum shall be 8 $\log_{10}$ CFU/ml.

2.3.2. Preparation of test specimens

Inoculate sterile cotton (1 cm^2) samples with 20 µl *E. faecium* inoculum suspension in a sterile petri dish, and allow to dry in the dark at room temperature for 18 hours before use.
For each chemical tested, 10 samples will be required; two test specimens are sealed within separate test enclosures for chemistry test wash; two test specimens are sealed within separate test enclosures for control water only wash; two loose textile samples without an enclosure for chemistry test; two loose textile samples without an enclosure for water only control and two loose textile samples as untreated controls.

2.3.3. Antimicrobial Activity of Chemicals against Loose Textile Samples

a) Prepare solutions of each chemical in 10 ml sterile distilled water to the predetermined test concentration (2.2.3).
b) Immerse loose 1cm^2 textile samples in 10 ml chemical solution for 13 minutes at room temperature without shaking.
c) Water only control samples shall be performed simultaneously, where samples are immersed in 10 ml sterile distilled water only for 13 minutes.
d) Untreated control samples shall be performed simultaneously, where samples are used as prepared, not subject to water or any antimicrobial treatment.
e) Place each test and control textile sample in 30 ml neutraliser and shake by hand 30 times. Incubate at room temperature for 5 minutes to allow neutralization of the test chemical to occur.
f) After 5 minutes of neutralization, briefly mix using a vortex mixer before transferring 1 ml test solution into 9 ml PBS (10^{-1} dilution). Vortex 10^{-1} dilution and transfer 1 ml into 9 ml PBS (10^{-2} dilution).
g) In duplicate, pipette 100 µl of the undiluted neutraliser solution onto separate nutrient agar plates and spread over the surface with a sterile spreader.
h) Spread duplicate 1 ml samples of undiluted neutraliser onto separate nutrient agar plates with a sterile spreader.
i) Spread 100 µl of the 10^{-1} and 10^{-2} dilutions onto separate nutrient agar plates with a sterile spreader.
j) Filter the remaining neutraliser through a membrane filter (2.2.10), then aseptically transfer the filter onto a nutrient agar plate.
k) Incubate plates at 37°C for 48 hours. Count the number of colonies on plates with 15-330 CFU.
l) Tests should be performed on two separate occasions (total sample number = 4).

2.3.4. Effect of Test Enclosure on Antimicrobial Activity of Laundry Chemicals

a) Seal the contaminated cotton (1 cm^2) test specimens in the test enclosure. Place the enclosure in a sterile 50/50 polycotton pillowcase and seal with a plastic zip tie. Alternatively, attach the enclosure to a sterile ballast sheet using a plastic retail tag, ensuring that the tag only pierces a free edge and does not pierce the compartment containing the contaminated textile sample.
b) Place the enclosed test specimens in the washing machine along with the sterile ballast load (2.2.6).
c) Wash the test enclosures according to the wash cycle outlined in 2.2.9.
d) Add the appropriate dose of the test chemical to the main wash stage of the cycle that produces the concentration outlined in 2.3.3a according to the wash stage water volume.
e) Upon completion of the wash, remove the cotton test specimens from their enclosures. Place in 30 ml neutraliser and shake by hand 30 times. Incubate at room temperature for 5 minutes.
f) Prepare and plate a dilution series of the neutraliser and enumerate each sample, according to 2.3.3 (f-j).
g) Tests should be performed on two separate occasions (total sample number = 4).
h) A water only control test should also be performed using the same method.

2.3.5. Interpretation

a) Calculate the $\log_{10}$ CFU per textile sample for each condition according to equation 1:

$$Log_{10}\ CFU\ per\ sample = Log_{10}\ [(\frac{No.\ of\ colonies}{Dilution\ factor}) \times\ Volume\ of\ neutraliser)] \quad \text{(Eq. 1)}$$

Untreated controls should equal 10^6 CFU per sample.

b) Calculate the $\log_{10}$ reduction for each test chemical, and water only control, from the untreated control according to equation 2:

$$Log_{10}\ Reduction = Log_{10}\ CFU\ per\ sample\ (untreated\ control) - Log_{10}\ CFU\ per\ sample\ (test) \quad \text{(Eq. 2)}$$

c) Calculate the difference between each test chemical and the water only control (LgW) according to equation 3:

$$LgW = Log_{10}\ Reduction\ (test) - Log_{10}\ reduction\ (water\ only\ control) \quad \text{(Eq. 3)}$$

d) Calculate the difference in LgW (ΔLgW) for each chemical between loose textile samples (2.3.3) and textile samples sealed in the test enclosure (2.3.4) according to equation 4:

$$\Delta LgW = LgW\ (loose\ textile\ sample) - LgW\ (enclosed\ sample) \quad \text{(Eq. 4)}$$

A negative ΔLgW indicates a reduction in antimicrobial activity of the chemical against *E. faecium* in the enclosure and a positive ΔLgW suggests greater activity against enclosed samples than loose samples.

e) The membrane is considered permeable at the test temperature where the ΔLgW is greater than -1.0 against all test chemicals.

f) Where the membrane is found to be impermeable at ambient temperature, tests may be repeated with elevated test temperatures (2.2.9). Laundry validation tests may only be conducted at the minimum temperature that the membrane is shown to be permeable.

g) The membrane must also satisfy Test 2 below to qualify for use in validation tests.

3. Test 2: Retention of *E. faecium* by Test Enclosure During Laundering

3.1. Principle

The test involves ensuring that the *E. faecium* on the cotton sample is not escaping the sealed membrane during the wash process. Bioindicators are prepared and washed in a defined wash programme in the presence of polycotton makeweights. The number of *E. faecium* cells retained within the bioindicator after washing is calculated and compared to the control with a $\log_{10}$ reduction per textile sample of <0.5 indicating the enclosure retains *E. faecium* during the wash process.

3.2. Materials

3.2.1. Microorganisms

Enterococcus faecium NCIMB 2699 (2.2.1).

3.2.2. Nutrient agar (2.2.2)

3.2.3. Diluent

PBS (2.2.4).

3.2.4. PBS-T .

PBS supplemented with 2 g/L polysorbate 80, prepared with distilled water and sterilised by autoclaving.

3.2.5. Textile

Woven 100% cotton, 1 cm^2 (2.2.5).

3.2.6. Washing machine ballast load (2.2.6)

3.2.7. Test Enclosure (2.2.8)

3.2.8. Washing Machine

A washer extractor equipped with a cold wash cycle consisting of a 4-minute pre-wash, 10-minute main wash and three rinse cycles of 2 minutes each should be used (2.2.9).

3.2.9. Membrane filters and filtration unit (2.2.10)

3.3. **Test Method**

3.3.1. Inoculum preparation

As per Test 2.3.1

3.3.2. Preparation of test specimens

As per Test 2.3.2.

For each enclosure to be tested, two samples are tested. Prepare a further 2 inoculated test swatches to be washed loose in the test wash (no membrane control) and 2 to be used as untreated controls.

3.3.3. Wash Test of Prepared Samples

a) Seal the contaminated cotton test specimens in the test enclosure. Place the enclosure in a sterile 50/50 polycotton pillowcase and seal with a plastic zip tie. Alternatively, attach the enclosure to a sterile ballast sheet using a plastic retail tag, ensuring that the tag only pierces a free edge and does not pierce the compartment containing the contaminated textile sample. Include a further 2 inoculated test swatches (no membrane controls) in the pillowcase or attached to a sterile ballast sheet.

b) Place the test samples in the washing machine with 2 kg sterile polycotton makeweights.
c) Wash at ambient temperature (3.1.8) with water only.
d) Untreated controls should be kept at ambient temperature without treatment during the wash cycle.
e) Aseptically recover the test samples from the washing machine, Remove the textile sample from the test membrane and place the textile samples in 30 ml PBS-T and shake by hand 30 times. Also place the no membrane controls and untreated controls into 30 ml PBS-T and shake by hand 30 times. Incubate at room temperature for 5 minutes.
f) Prepare and plate a dilution series of PBS-T and enumerate each sample, according to Tests 2.3.3 (f-j).
g) Tests should be performed on two separate occasions (total sample number = 4).

3.3.4. Interpretation

a) Calculate the $\log_{10}$ CFU per textile sample for each condition according to equation 5:

$$Log_{10}\ CFU\ per\ sample = Log_{10}\ [(\frac{No.\ of\ colonies}{Dilution\ factor}) \times\ Volume\ of\ PBS - T)] \quad \text{(Eq. 5)}$$

Untreated controls should equal 10^6 CFU per sample.

b) Calculate the $\log_{10}$ reduction for each sample from the untreated control according to equation 6:

$$Log_{10}\ Reduction = Log_{10}\ CFU\ per\ sample\ (untreated\ control) - Log_{10}\ CFU\ per\ sample\ (test) \quad \text{(Eq. 6)}$$

c) A $\log_{10}$ reduction per textile sample of <0.5 indicates that the enclosure retains *E. faecium* during the wash process.
d) Test 1 must also be satisfied in order for the enclosure to be used for validation of wash processes.

4. Test 3: Validation of Neutralisers for Additional Chemicals

4.1. Principle

A neutraliser validation method was adapted from the BS EN 1040:2005 neutralisation-dilution validation tests (British Standards Institution, 2005), using inoculated cotton swatches in place of microbial suspensions.

4.2. Materials

4.2.1. Microorganisms

Enterococcus faecium NCIMB 2699 (Test 2.2.1).

4.2.2. Nutrient agar (2.2.2)

4.2.3. Diluent

PBS (2.2.4)

4.2.4. Distilled water

Freshly distilled water, sterilised by autoclaving.

4.2.5. Test chemical

Additional disinfectant chemical to be assessed (Test 2.2.3)

4.2.6. Neutraliser

A suitable neutraliser shall be selected according to the test chemical; examples of suitable neutralisers for disinfectant chemicals are listed in Test 1 2.2.3 or BS EN 13727:2012+A2:2015 Annex B. The neutralizer shall be adjusted to pH 7.2 and sterilized according to suitable means.

4.2.7. Textile

Woven 100% cotton, cut into 1 cm^2 samples and sterilised by autoclaving.

4.3. Test Procedure

4.3.1. Inoculum preparation

As per Test 2.3.1

4.3.2. Preparation of test specimens

For each chemical to be assessed, two test specimens should be tested in parallel and the test should be repeated on another occasion (n=4).

As per Test 2.3.2.

4.3.3. Neutraliser Toxicity

a) Immerse the test specimen (Test 4.3.2.) in 30 ml neutraliser (4.2.6) and shake by hand 30 times.
b) Incubate at room temperature for 5 minutes.
c) After neutralisation for 5 minutes, briefly mix the sample before serially diluting in PBS and enumerating using nutrient agar (Tests 2.3.3 f-j).
d) **Untreated Control:** Immerse a separate test specimen in 30 ml sterile distilled water (Test 4.2.4) and process as per steps b-c.

4.3.4. Neutraliser Efficacy

a) Dispense 1 ml of the test chemical (Tests 4.2.5) into 29 ml neutraliser (Test 4.2.6).
b) Vortex mix the samples and incubate for 5 minutes at room temperature to allow neutralisation to occur.
c) After the 5 minutes of neutralisation, briefly vortex mix again
d) Add the test specimen (Test 4.3.2.), incubate 30 minutes at room temperature.
e) Shake by hand 30 times before serially diluting in PBS and enumerating using nutrient agar (Tests 2.3.3 f-j).
f) **Water Only Control:** Dispense 1 ml of sterile distilled water (Test 4.2.4) into 29 ml neutraliser (Test 4.2.6) and process as per steps b-e.

4.3.5. Interpretation

4.2.5.1 Neutraliser Toxicity

a) Calculate the $\log_{10}$ CFU per textile sample for the test and untreated control samples according to equation 7:

$$Log_{10}\ CFU\ per\ sample = Log_{10}\ [(\frac{No.\ of\ colonies}{Dilution\ factor}) \times\ 30\ ml)] \qquad \text{(Eq. 7)}$$

Untreated controls should equal 10^6 CFU per sample.

b) Calculate the neutralizer toxicity value according to equation 8:

$$Neutraliser\ Toxicity\ Value = Log_{10}\ CFU\ per\ sample\ (untreated\ control) - Log_{10}\ CFU\ per\ sample\ (test\ sample) \qquad \text{(Eq. 8)}$$

The neutraliser is considered non-toxic where the neutraliser toxicity value is ≤0.5.

4.2.5.2 Neutraliser Efficacy

a) Calculate the $\log_{10}$ CFU per textile sample for the test and water only control samples according to equation 9:

$$Log_{10}\, CFU\ per\ sample = Log_{10}\, [(\frac{No.\ of\ colonies}{Dilution\ factor}) \times\ 30\ ml)] \qquad \text{(Eq. 9)}$$

b) Calculate the neutralizer efficacy value according to equation 10:

$$Neutraliser\ Efficacy\ Value = Log_{10}\, CFU\ per\ sample\ (water\ only\ control) - Log_{10}\, CFU\ per\ sample\ (test\ sample) \qquad \text{(Eq. 10)}$$

The neutraliser is considered effective where the neutraliser efficacy value is ≤0.5.

Supplementary Material

5. Preparation of *E. faecium* Bioindicators for Use in Test One: Microbicidal Activity of Wash Process – Validation using Bioindicators (Annex I) & Preparation and Validation of Bioindicator Enclosures for Use in Industrial Laundry Disinfection Process Test (Annex II)

5.1. Materials

5.1.1. Microorganisms

E. faecium NCIMB 2699 (Test 2.2.1).

5.1.2. Diluent

PBS (3.3.2).

5.1.3. Textile

Woven 100% cotton, 1 cm^2 (Test 2.2.5).

5.1.4. Enclosure Membrane

To be validated according to Tests 1 and 2. The membrane shall be sterilised by suitable means and of a suitable size and configuration to seal a1 cm^2 cotton sample in a compartment. If multiple samples of the same or different log_{10} inoculations are to be tested at the same time, multiple compartments can be created. Polyethersulfone (PES) membranes with a pore size of 0.22µm have been previously validated by Owen *et al*. (2024).

5.1.5. Heat sealer

Any given heat sealer should deliver enough heat to be able to seal two layers of the membrane together.

5.2. Preparation of In-House Bioindicators

5.2.1. Inoculum preparation

a) In duplicate, prepare test suspensions of *E. faecium* (10^8 CFU/ml*)* according to Test 2.3.1.
b) Serially dilute the test suspension in PBS to produce an inoculum concentration (CFU/ml) from which 20 µl will produce the required microbial load on the cotton sample. For a 10^6 textile sample load a concentration of 10^8 CFU/ml will be needed and for a 10^5 textile sample load a concentration of 10^7 CFU/ml will be required. After inoculation and drying (5.2.2 a & b) microbial load on the textile sample should

be assessed because of potential microbial death resulting in the initial inoculum concentration needing adjusting accordingly.

5.2.2. Preparation of test specimens

a) Inoculate two 1 cm^2 cotton samples with 20 µl of prepared inoculum (5.2.1b) to produce the required microbial load on the textile sample. One set of samples will be laundered and one set of samples will be used as an unlaundered control.
b) Allow to dry in the dark at room temperature for 18 hours before use.
c) Seal one of the contaminated cotton samples within the enclosure. The compartment formed should be > 2cm x 2cm in size not including the sealed edge.

References

1. BS EN 13727:2012+A2:2015 - 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).
2. BS EN ISO 6330:2021 – Textiles – Domestic washing and drying procedures for textile testing.
3. BS EN 1040:2005 - Chemical disinfectants and antiseptics - Quantitative suspension test for the evaluation of basic bactericidal activity of chemical disinfectants and antiseptics - Test method and requirements (phase 1).
4. Owen, Lucy, Caroline Cayrou, Georgina Page, Martin Grootveld, and Katie Laird. (2024). "Development of a Standardised International Protocol for Evaluation of the Disinfection Efficacy of Healthcare Laundry Wash Processes" *Applied Microbiology* 4, no. 1: 194-214. https://doi.org/10.3390/applmicrobiol4010014