The linearity of an alternative quantitative method is its ability (within a given range) to produce results that are proportional to the concentration of micro-organisms present in the sample. The linearity must be determined over a reasonable range (e.g. \(10^2-10^6\) CFU/mL) so as to correspond to the purpose of the alternative method. One approach would be to select different concentrations of each test micro-organism and test several replicates. For each concentration, an appropriate number of replicates is chosen to confirm linearity. The number of replicates is chosen so that the entire test can be carried out during the same working session. After checking the homogeneity of the variances of the results obtained for each concentration, the regression line is calculated. Linearity is demonstrated if the estimated slope is significant and if the test for deviation from linearity is non-significant (see general chapter 5.3).

3-3-2-6. Range

The range of an alternative quantitative method is the interval between the upper and lower levels of micro-organisms as determined from the related studies of precision, accuracy and linearity using the specified method; it is dependent on the intended application.

3-3-2-7. Robustness

The robustness of an alternative quantitative method is a measure of its capacity to remain unaffected by small but deliberate variations in method parameters (e.g. incubation period or incubation temperature range). Robustness is a validation parameter best suited to determination by the supplier of the method. Nevertheless, if the user modifies critical parameters, the effects on robustness must be evaluated. Robustness of an alternative quantitative method is judged by its ability to correctly enumerate the test micro-organisms after deliberate variations to the method parameters.

3-3-2-8. Suitability testing

The alternative method must be applied according to the specified procedure and with the samples to be analysed under the responsibility of the user. It must be shown that the test sample does not interfere with the system's enumeration capacity or microbial recovery. Specific points to be addressed are:

- the ability of the test to detect micro-organisms in the presence of the sample matrix;
- verifying if the sample matrix interferes with the alternative system (e.g. background signal or inhibiting chemical reactions).

Acceptance criteria for the method are defined as a function of the application and of the validation data.

3-3-2-9. Equivalence testing

Equivalence testing of 2 quantitative methods can be conducted directly on the validation parameters. This approach requires an adequate comparison experiment at low levels of inoculation (e.g. less than 5 CFU) with sufficient numbers of replicates for relevant strains of test micro-organisms. Alternatively, and in some cases additionally, equivalence testing can be carried out by the parallel testing of a predefined number of samples or for a predefined period of time. This parallel testing can be justified based on a risk assessment.

If the result of the alternative method can be expressed as the number of CFUs per weight or per volume, statistical analysis of the results shall demonstrate that the results of the alternative method enable an unequivocal decision as to whether compliance with the standards of the monographs would be achieved if the official method was used.

If the result of the alternative method cannot be expressed as the number of CFUs, equivalence testing is performed using suitable parameters, followed by statistical analysis to demonstrate that the results of the alternative method enable an unequivocal decision as to whether compliance with the standards of the monographs would be achieved if the official method was used.

3-3-3. Validation of alternative identification tests

There is a large body of evidence that different methods vary considerably in their ability to identify micro-organisms. It must be accepted that a method of identification needs to be internally consistent, but may differ from others in its identification of micro-organisms.

3-3-3-1. Accuracy

The accuracy of an alternative identification method is its ability to identify the desired micro-organism to the required taxonomic level. It must be demonstrated using well-characterised reference micro-organisms, e.g. type strains. Accuracy of the identification method is usually expressed as the number of correct identifications divided by the total number of identifications.

3-3-3-2. Specificity

The specificity of an alternative identification method is its ability to discriminate micro-organisms actually present from interfering factors that cause false identification results. Such factors include chemical substances and mixtures of micro-organisms, which cause the test to identify micro-organisms not actually present in the sample material (e.g. the presence of mixtures of DNA material from 2 micro-organisms in a sequencing test leading to the false identification of a third micro-organism).

3-3-3-3. Robustness

The robustness of an alternative identification method is a measure of its capacity to remain unaffected by small but deliberate variations in method parameters (e.g. incubation period or incubation temperature range). Robustness is a validation parameter best suited to determination by the supplier of the method. Nevertheless, if the user modifies critical parameters, the effects on robustness have to be evaluated. Robustness of an identification method is judged by its ability to correctly identify the test micro-organisms after deliberate variations to the method parameters.
bactericidal activity and 15 min for fungicidal or yeasticidal activity. Additional contact times may be chosen, according to the stated use of the antiseptic medicinal product. At the end of the contact time, an aliquot is taken and the antimicrobial activity in this aliquot is immediately stopped by a validated method. 2 methods are available: dilution-neutralisation and membrane filtration.

The procedure is validated to verify its ability to demonstrate the required reduction in the count of viable micro-organisms by the use of appropriate controls.

2. TEST MICRO-ORGANISMS AND GROWTH CONDITIONS

Prepare standardised stable suspensions of test strains as stated in section 2-1. Seed-lot culture maintenance techniques (seed-lot systems) are used so that the viable micro-organisms stated in section 2-1. Seed-lot culture maintenance techniques Prepare standardised stable suspensions of test strains as described in Table 5.1.11.-1.

From the original master seed-lot. Grow each of the microbial (seed-lot systems) are used so that the viable micro-organisms

The recommended solutions and media are described in general chapter 2.6.13. Purified water is used. All reagents are sterile prior to use.

The test for bactericidal, fungicidal or yeasticidal activity is performed with the designated strains as described in Table 5.1.11.-1. In addition to these micro-organisms, it may be necessary to add other bacterial or fungal strains that represent the indications of the antiseptic medicinal product tested.

Single-strain challenges are used. The counts are performed in duplicate and the arithmetic mean of the results is calculated and expressed in CFU/mL.

1-1. PREPARATION OF TEST SUSPENSION

For harvesting the micro-organisms use a sufficient volume of a 9 g/L solution of sodium chloride R (for bacteria and C. albicans) or a solution containing 9 g/L of sodium chloride R and 0.5 g/L of polysorbate 80 R (for A. brasiliensis), to obtain a test suspension with the number of CFU described in Table 5.1.11.-1. Use the suspension within 2 h or within 24 h if stirred at 2-8 °C.

2-2. PREPARATION OF ANTISEPTIC PRODUCT TEST SOLUTION

The concentration of the antiseptic product test solution shall be, if possible, 1.25 times the in-use test concentration because it is diluted to 80 per cent during the test and the method validation.

2-3. NEUTRALISING AGENTS

Neutralising agents are used to neutralise the antimicrobial activity of the antiseptic product. The common neutralising agents are listed in Table 2.6.12.-2 of general chapter 2.6.12. Microbiological examination of non-sterile products: microbial enumeration tests. The neutralisation time is not less than 10 s and not more than 60 s.

3. METHODS

Prior to testing, equilibrate the temperature of all reagents to 33 ± 1 °C.

3-1. DILUTION-NEUTRALISATION METHOD

Transfer 1.0 mL of a 3 g/L solution of bovine albumin R into a tube, add 1.0 mL of the test suspension and maintain at 33 ± 1 °C for 5 min. Add 8.0 mL of the antiseptic product test solution and maintain at 33 ± 1 °C for the chosen contact time. Then, take a 1.0 mL sample of the test mixture and transfer into a tube containing 1.0 mL of water R and 8.0 mL of the neutralising agent and maintain at 33 ± 1 °C for the appropriate neutralisation time. Take 1.0 mL of the neutralised test mixture, in duplicate, and inoculate using the pour-plate or surface-spread method. For incubation conditions, see Table 5.1.11.-1. After incubation, perform the count.

3-1-1. Suitability of the test/controls

For all methods, prepare a validation suspension containing 100-1000 CFU of the test micro-organisms per millilitre.

3-1-1-1. Experimental conditions control

Transfer 1.0 mL of a 3 g/L solution of bovine albumin R into a tube, add 1.0 mL of the validation suspension and maintain at 33 ± 1 °C for 2 min. Add 8.0 mL of water R and maintain at 33 ± 1 °C for the chosen contact time. Take 1.0 mL of this mixture, in duplicate, and inoculate using the pour-plate or surface-spread method. For incubation conditions, see Table 5.1.11.-1. After incubation, perform the count. The number of CFU recovered following incubation is not less than 0.5 × (number of CFU in the validation suspension)/10.

3-1-1-2. Neutralising agent control

Transfer 1.0 mL of a 3 g/L solution of bovine albumin R into a tube, add 1.0 mL of the validation suspension and 8.0 mL of the neutralising agent used in the test and maintain at 33 ± 1 °C for the appropriate neutralisation time. Take 1.0 mL
of this mixture, in duplicate, and inoculate using the pour-plate or surface-spread method. For incubation conditions, see Table 5.1.11.-1. After incubation, perform the count. The number of CFU recovered following incubation is not less than 0.5 × (number of CFU in the validation suspension)/10.

3-1-1-3. Dilution-neutralisation method control

Transfer 1.0 mL of a 3 g/L solution of bovine albumin R into a tube, add 1.0 mL of a 9 g/L solution of sodium chloride R and 8.0 mL of the product test solution and maintain at 33 ± 1 °C for the chosen contact time. Transfer 1.0 mL of this mixture into a tube containing 8.0 mL of the neutralising agent and maintain at 33 ± 1 °C for the appropriate neutralisation time. Then add 1.0 mL of the validation suspension and mix. After 30 min, take a sample of 1.0 mL of the mixture, in duplicate, and inoculate using the pour-plate or surface-spread method. For incubation conditions, see Table 5.1.11.-1. After incubation, perform the count. The number of CFU recovered following incubation is not less than 0.5 × (number of CFU in the validation suspension)/10.

3-2. MEMBRANE FILTRATION METHOD

Proceed as described in section 3-1, carrying out immediately the filtration step in place of the neutralisation step.

Use membrane filters having a nominal pore size not greater than 0.45 μm. The type of filter material is chosen such that the microbe-retaining efficiency is not affected by the components of the sample to be investigated. For each of the micro-organisms listed, a single membrane filter is used. Appropriately dilute 0.1 mL of the test solution and immediately filter the total volume, then rinse the membrane filter with an appropriate volume of the diluent. Perform the test in duplicate. For incubation conditions, see Table 5.1.11.-1. After incubation, perform the count.

3-2-1. Verification of the selected experimental conditions and of the membrane filtration method

3-2-1-1. Experimental conditions control

Proceed as described in section 3-1-1-1, except at the end of the contact time, take the sample in duplicate, and transfer into a separate membrane filtration apparatus. Filter immediately and then transfer each of the membrane filters to the surface of separate plates. For incubation conditions, see Table 5.1.11.-1. After incubation, perform the count. The number of CFU recovered following incubation is not less than 0.5 × (number of CFU in the validation suspension)/10.

3-2-1-2. Membrane filtration method control

Proceed as described in section 3-1-1-3, except at the end of the chosen contact time, take that sample in duplicate, and transfer into a separate membrane filtration apparatus. Filter and rinse as described in section 3-2, then cover the membranes with rinsing liquid and add a sample of the validation suspension. Filter again and transfer each of the membrane filters to the surface of separate plates. For incubation conditions, see Table 5.1.11.-1. After incubation, perform the count. The number of CFU recovered following incubation is not less than 0.5 × (number of CFU in the validation suspension)/10.

4. ACCEPTANCE CRITERIA

Unless otherwise justified and authorised, the preparation has a:
- bactericidal activity if the defined number of CFU is reduced by at least 5 \( \log_{10} \);
- fungicidal activity if the defined number of CFU is reduced by at least 4 \( \log_{10} \);
- yeasticidal activity if the defined number of CFU is reduced by at least 4 \( \log_{10} \).