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 2 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 $\log_{10}$.

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5.1.12. DEPYROGENATION OF ITEMS USED IN THE PRODUCTION OF PARENTERAL PREPARATIONS

This general chapter covers pyrogen inactivation or removal from items (primary packaging materials and equipment) coming into direct contact with the final sterilised product.

Pyrogens are substances that have the ability to induce fever when infused or injected into the body.

In this general chapter, depyrogenation is defined in terms of a reduction in lipopolysaccharide (endotoxin), the most potent and difficult-to-eliminate of pyrogenic materials.

1. DEPYROGENATION PROCESSES

1-1. *DRY HEAT TREATMENT*

1-1-1. **Treatment conditions**

Dry heat treatment is the most common depyrogenation process for glassware and other non-heat-labile materials.

A common dry heat depyrogenation cycle consists of subjecting the items to a temperature of at least 250 °C for at least 30 min; other validated combinations of temperature and time may be used, but the minimum combination required is 180 °C for 3 h.

1-1-2. **Equipment**

Dry heat treatment is carried out in an oven or tunnel with forced air circulation or using other equipment specifically designed for this purpose.

As a minimum, relevant dry heat depyrogenation parameters (e.g. time, temperature and, where applicable, belt speed) are monitored at the most difficult-to-heat position in the chamber, which is defined during the qualification study on the equipment.

1-1-3. **Validation**

All processes must be validated by adding suitable endotoxin indicators to the load at the positions identified as being the most difficult to heat (and therefore to depyrogenate) in order to verify the efficacy of depyrogenation.

1-2. *OTHER PROCESSES*

Other processes may be used only when heat treatment is not possible.

1-2-1. **Physical treatment**

Depyrogenation may also be achieved by rinsing the items with an appropriate pharmaceutical-grade water such as water for injections (WFI), with or without additional physical means.

Items must be dried following cleaning.

1-2-2. **Chemical treatment**

Chemical reactions using strong oxidative, alkylating or reducing agents, either liquids or vapour-phase gases, with or without heat, capable of removing pyrogens, may be applied and followed by final rinses with WFI. At the end of the treatment a verification test is carried out to detect any chemical residues.

1-2-3. **Validation**

All processes must be validated by adding suitable endotoxin indicators to the load at the positions identified as being the most difficult to depyrogenate in order to verify the efficacy of depyrogenation.

2. CHARACTERISTICS OF ENDOTOXIN INDICATORS

Endotoxin indicators consist of purified bacterial endotoxins (lipopolysaccharides). The following are required to be known by the user:

- name of the producer;
- composition of the indicator system if applicable;
- genus and species of the micro-organism (including the culture collection number if available) from which the endotoxin is isolated;
- specific activity of the endotoxin, in International Units per gram, per millilitre or per container;
- storage conditions and expiry date;
- batch number.

Depending on the nature of the item to be depyrogenated (e.g. glass, stainless steel, plastic), a suitable endotoxin indicator (ready-to-use or custom-made) is used during validation of a depyrogenation process.

2-1. *READY-TO-USE ENDOTOXIN INDICATORS*

These indicators are ampoules, vials or other carriers that have been inoculated with a known amount of endotoxin and are used to measure endotoxin reduction or inactivation in a depyrogenation process.

The type of carrier used must be representative of the item that is being depyrogenated as this may influence the effectiveness of the depyrogenation process.

2-2. *CUSTOM-MADE ENDOTOXIN INDICATORS*

Custom-made endotoxin indicators are items (e.g. primary packaging materials or equipment) that have been surface-inoculated with a suitable endotoxin solution (e.g. endotoxin reference standard). Once dried, the inoculated item is used to measure endotoxin reduction or inactivation in a depyrogenation process.

3. TEST PROCEDURE

3-1. *RECOVERY TEST PROCEDURE OF ENDOTOXIN FROM ENDOTOXIN INDICATORS*

The endotoxin recovery rate is determined on items (ready to use or custom made indicators) that are not subjected to the depyrogenation process in order to calculate the percentage recovery and to check any interference from leachables before assessing the effectiveness of a depyrogenation process.

The endotoxin recovery test must be validated.

If the endotoxin recovery rate is insufficient to demonstrate a 3.0 $\log_{10}$ reduction, the amount of endotoxin used must be adjusted accordingly.

3-2. *ENDOTOXIN REDUCTION TEST PROCEDURE*

Items that have been artificially contaminated with a known amount of endotoxin or ready-to-use endotoxin indicators are placed in the load to be tested. Endotoxin levels are assessed before (initial endotoxin level) and after (residual endotoxin level) the depyrogenation process according to the same validated method. The level of endotoxin per indicator and the number of indicators used for the test must be sufficiently high to allow accurate determination of the reduction in endotoxin due to the depyrogenation process.

4. CRITERIA

Not less than a 3.0 $\log_{10}$ reduction in recoverable endotoxin is used as the criterion for process validation. The $\log_{10}$ reduction is calculated by determining the measured $\log_{10}$ of recoverable endotoxin in the unprocessed item minus the measured $\log_{10}$ of recoverable endotoxin remaining in the processed item.