Australian Government
Department of Health and Ageing
Therapeutic Goods Administration

TGA guidelines for sterility testing of therapeutic goods

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TGA Health Safety Regulation

About the Therapeutic Goods Administration (TGA)

- The TGA is a division of the Australian Government Department of Health and Ageing, and is responsible for regulating medicines and medical devices.
- TGA administers the *Therapeutic Goods Act 1989* (the Act), applying a risk management approach designed to ensure therapeutic goods supplied in Australia meet acceptable standards of quality, safety and efficacy (performance), when necessary.
- The work of the TGA is based on applying scientific and clinical expertise to decision-making, to ensure that the benefits to consumers outweigh any risks associated with the use of medicines and medical devices.
- The TGA relies on the public, healthcare professionals and industry to report problems with medicines or medical devices. TGA investigates reports received by it to determine any necessary regulatory action.
- To report a problem with a medicine or medical device, please see the information on the TGA website.

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1. INTRODUCTION

100. Relevant section of the BP/Ph Eur: 'Guidelines for Using the Test for Sterility'

Regulatory aspects

101. The *TGA Guidelines on Sterility Testing of Therapeutic Goods* provide guidance for sterility testing of sterile therapeutic drugs and devices supplied in Australia for human use. They are intended for use by manufacturers and the Official Analysts of the Therapeutic Goods Administration (TGA) Laboratories, and as guidance for referee testing when results are in dispute. Some sections therefore include information relating to referee testing.

102. It should be noted that these Guidelines are not mandatory for industry. The official (legal) requirements for sterility tests in Australia are currently those specified in the most recently gazetted *British Pharmacopoeia* (BP), which are aligned with the *European Pharmacopoeia* (Ph Eur). Therapeutic Goods Order Number 11 *Standard for Sterile Therapeutic Goods* was revoked on the date of adoption of the BP 1998. The BP, Ph Eur and *US Pharmacopoeia* (USP) sterility test methods became harmonised with the publication of the BP 2004, Ph Eur 5.1 and USP 28 editions and as such all are considered to be acceptable by the TGA.

103. These Guidelines are based on the superseded document *Australian Code of Good Manufacturing Practice for Therapeutic Goods - Medicinal Products - Appendix C Guidelines on Tests for Sterility,* 1990 (Appendix C). They also incorporate the BP/Ph Eur requirements with additional elements from the Pharmaceutical Inspection Convention/Pharmaceutical Inspection Co-operation Scheme (PIC/S) *Recommendations on Sterility Testing,* the USP and the combined experience of the TGA Laboratories, manufacturers and contract testing laboratories.

104. Sampling schedules and specific guidance for testing of medical devices are provided because these are not included in the BP/Ph Eur. Sampling schedules are based on those of the USP 28 effective January 2005.

105. Where the Guidelines describe particular procedural steps and media, it should be understood that other procedures and other media may be equally satisfactory, provided that their use can be validated. However, when the results of a sterility test performed by the TGA Official Analyst are challenged, the combined procedures of the BP/Ph Eur test and this document must be followed.

106. Where reference is made to the 'competent authority' throughout this document, the TGA fulfils this role in Australia.

Rationale

107. Success in detecting microbial contamination of goods is dependent *inter alia* upon statistical considerations. Generally, the odds are against detecting a low level of contamination, ie, when relatively few organisms contaminate a small proportion of items. Detection of contamination with absolute certainty would necessitate the examination of all items of the batch using media capable of supporting the growth of all possible contaminants.

108. In the BP/Ph Eur sterility test prior to 1998, when contamination was found in a primary test, a repeat test was permitted as a check against the possibility that the contamination was introduced by the operator and was not a contamination of the material tested. Repeat testing reduces the efficiency of testing because the probability of accepting a contaminated batch is thereby increased.

109. The probability of including contaminated items in two successive samples taken from a given batch is the product of the probabilities of a contaminated item being included in each of the single samples. For example, with a sample size of 10 and a contamination rate of 5% the probability of including a contaminated item is 0.4 (see Table 1).

110. If the test is repeated with another sample size of 10 units the probability of including a contaminated item is again 0.4. However, the probability of both tests being positive is 0.4 x 0.4 = 0.16 which is lower than the probability of the first test, and hence in 84 of 100 such tests the batch will be accepted as sterile.

TABLE 1: PROBABILITIES OF DETECTING A CONTAMINATED LOT IN A SINGLE TEST

Percentage of Items contaminated	0.1	1.0	2.0	5.0
Sample size 10	0.01	0.09	0.18	0.40
Sample size 20	0.02	0.18	0.33	0.65
Sample size 50	0.05	0.39	0.64	0.92
Sample size 100	0.09	0.63	0.87	0.99

111. A repeat test is now permitted only if it can be clearly demonstrated that the test was invalid for causes unrelated to the product being examined (see clauses 492-496).

112. Sterility cannot be guaranteed by the quality control laboratory. It must be built into the product during processing. Experience in many countries over the years has confirmed that greater reliance must be placed on appropriate techniques and procedures throughout the manufacture of the product (including in-process sterility testing at various stages) rather than simply depending on sterility tests made on a number of samples of the final batch as the sole criterion of sterility.

113. Permission to delete the sterility test from batch release specifications may be granted by the competent authority where manufacturing standards are of a high order and the product is terminally sterilised in its final container.

114. In all other cases, sterility testing is necessary to detect contamination arising from technical malfunction, human error or mix-up between sterilised and non-sterilised goods. It must be performed as part of batch release specifications for product that is not terminally sterilised in the final container. It is also the only analytical method available for finished product testing by the competent authority.

115. It is important to realise that background contamination as detected by negative controls can obscure low levels of product contamination and for this reason every effort should be made to ensure that background contamination is kept as low as possible. Statistics compiled by the TGA Laboratories show that skilled operators working under the prescribed conditions can achieve a level as low as one contamination in five thousand control inoculations (0.02%).

2. SAMPLING OF LOTS

200. Relevant sections of the BP/Ph Eur: 'Application of the test to parenteral preparations, ophthalmic and other non-injectable preparations required to comply with the test for sterility'; 'Guidance to manufacturers'; Tables 2.6.1.-2 and 2.6.1.-3.

201. The sampling schedules in Tables 2 and 3 set out the minimum number of items to be sampled from each batch (lot) and the minimum quantity to be tested from each container, unless otherwise justified and authorised. The batch size and conditions of manufacture should be considered when planning a sampling regimen and larger numbers and quantities may be appropriate. It is assumed that the product has been manufactured under conditions designed to exclude contamination.

202. For the purposes of these Guidelines a batch of product is defined as a homogeneous collection of sealed containers prepared in such a manner that the risk of contamination is the same for each of the units of the batch. Where a batch is sterilised as a series of lots or sub-batches, each of which is subjected to a separate sterilising cycle or is subjected in processing to different treatment which may affect its sterility eg different freeze-drying cycles, each lot should be tested for sterility.

203. Any samples used for a sterility retest should reflect the original samples in terms of sampling locations or process times.

204. For aseptically prepared products, the samples should be taken at regular intervals during the filling operations in such a way that every filling point is represented by an approximately equivalent number of samples. Further, the first and last items dispensed at each filling point and the first item filled after any machine break-down or change, any non-validated intervention or interruption, should be included amongst the samples. Items selected as samples need not be discarded if they are under-filled provided they contain sufficient volume of product for the test. In the event of contamination being detected it is useful if the source and place in the filling run of samples can be identified.

205. For terminally sterilised products the samples should be made up from units drawn from various sites throughout the steriliser load. Some of the units should be taken from that place in the load known to be least accessible to the sterilising agent. Samples may be taken representatively from across each load, if the conditions for filling the containers were such as to satisfy the conditions for aseptically filled containers or the design of the equipment, the specification of the sterilising cycles and the validation data for each class of product are acceptable to the competent authority.

206. The minimum number of items or quantity to be tested may be inappropriate for some products and a smaller sample may be tested with the approval of the competent authority.

207. The testing of products which are of high intrinsic value or which are intended for use in clinical trials and which are dispensed in small volumes or produced in small lots of less than 20 containers, may be combined with the filling procedure using the following method:

- a sterile membrane filter is incorporated in the filling line;
- the containers are filled and a number of containers are sampled, the number and manner of selection being as specified in clause 204 and Table 2;
- the sample containers are swirled so as to ensure that the product contacts the entire internal surface of the container, and then, using aseptic procedures, the containers are opened and the contents are emptied back into the reservoir for the filling line;
- the product is then refilled into fresh containers;
- the in-line membrane filter is removed and tested for sterility by dividing it into two approximately equal portions and testing one portion in Fluid Thioglycollate Medium (Medium 1) and the other in Soybean-Casein Digest Medium (Medium 2) (see clauses 475-479).

208. Where the goods to be tested are intended for laboratory use only, the test for sterility may be performed on smaller samples than those indicated above, where rational considerations preclude such large-scale tests.

209. When the test method is membrane filtration, whenever possible, the whole contents of the container should be tested, but not less than the quantities indicated in Table 3.

TABLE 2: SAMPLING SCHEDULE - MINIMUM NUMBER OF ITEMS TO BE TESTED FROM EACH BATCH[1]

Type of product	Number of items in the batch	Minimum number of items to be tested for each medium[2]
Injectable pharmaceuticals Injectable medical devices Ophthalmic and other non-injectable pharmaceuticals in single-dose containers Ophthalmic medical devices in single-dose containers	Not more than 100	10% of batch or 4 containers, whichever is the greater
	101-500	10 containers
	More than 500	2% of batch or 20 containers, whichever is the lesser
Ophthalmic and other non-injectable pharmaceuticals and medical devices not in single-dose containers	Not more than 200	5% of batch or 2 containers, whichever is the greater
	More than 200	10 containers
Bulk solid products	Not more than 4	Each container
	5-50	20% of batch or 4 containers, whichever is the greater
	More than 50	2% of batch or 10 containers, whichever is the greater
Pharmacy bulk packages of antibiotics	Less than 5g	20 containers
	Greater than or equal to 5g	6 containers
Solid medical devices	Not more than 100	10% of batch or 4 units, whichever is the greater
	101-500	10 units
	More than 500	2% of batch or 20 units, whichever is the lesser

NOTES

1. This Table incorporates Table 2.6.1-3 (BP and Ph Eur) and Table 3 (USP).
2. If the contents of one container are sufficient to inoculate the two media, this column gives the number of containers needed for both the media together.

TABLE 3: MINIMUM QUANTITY OF PRODUCT TO BE TESTED FROM EACH CONTAINER IN THE SAMPLE[1]

Type of product	Quantity of product in unit container	Minimum quantity/proportion to be used for each medium from each container[2]
Liquids	Less than 1 mL	The whole contents
	1 - 40 mL	Half the contents but not less than 1 mL
	41 - 100 mL	20 mL
	Greater than 100 mL	10% of the contents but not less than 20 mL
Antibiotic liquids	N/A	1 mL
Other preparations soluble in water or isopropyl myristate	N/A	The whole contents to provide not less than 200 mg
Insoluble preparations, creams and ointments to be suspended or emulsified	N/A	The whole contents to provide not less than 200 mg
Solids	Less than 50 mg	The whole contents
	50 - 299 mg	Half the contents but not less than 50 mg
	300 mg to 5 g	150 mg
	Greater than 5 g	500 mg
Solid medical devices	One or more dressings	100mg
	One or more devices	One whole device[3], cut/disassembled if necessary

NOTES

1. This Table incorporates Table 2.6.1-2 (BP and Ph Eur) and Table 2 (USP).
2. Unless otherwise justified and authorised
3. Refer also to clauses 452 - 455.

3. PRECAUTIONS AGAINST MICROBIAL CONTAMINATION

300. Relevant section of the BP/Ph Eur: 'Precautions against microbial contamination'

301. Tests for sterility are to be carried out by trained personnel using techniques and equipment which minimise the risks of accidental microbial contamination of the tests and of the testing environment.

302. Tests for sterility should be conducted in a clean-room environment that is equivalent to the standard of clean-room required for the aseptic manufacture of pharmaceutical products. Additional guidance is given in Annex IV.

303. Personnel occupying the aseptic testing area during sterility testing or associated aseptic manipulations should wear sterilised overgarments. The use of sanitised garments may be acceptable under certain conditions (see Annex IV).

304. All equipment, vessels and materials with which the sterile test media or the goods under test may come into contact in the course of the testing should be sterilised prior to use. Preferred methods are heating in an autoclave so that all surfaces are held at a temperature of 121°C and exposed to saturated steam for at least 15 minutes, or by heating to, and holding for at least 2 hours at a temperature of 160°C in a hot air oven, or by exposure to a minimum absorbed radiation dose of 25 kGy.

305. All substances added to the goods tested, and all substances added to sterile media or introduced into sterile membrane filtration units (other than the preparations under test) should be sterilised prior to use by heat. If this reduces the effectiveness of the test the most effective alternative method should be used.

306. Prior to sterilisation, all vessels, substances or outer clothing to be used for the performance of tests for sterility, or introduced into the testing area, should be appropriately packaged or closed, so as to prevent access of micro-organisms. Each package or item being sterilised should bear a visual indicator appropriate to the method of sterilisation to indicate that it has been processed but the appropriate change in the appearance of the indicator should not be taken as a guarantee of the sterility of the contents. Each package or item should be dated with the date of sterilisation to assist in correct stock rotation.

307. The outer surfaces of all packages of equipment, vessels, etc., which are introduced into the aseptic testing environment (including vessels of media and packages or containers of goods to be tested) should be free of contamination immediately prior to their introduction into the aseptic environment: they should be sterilised or disinfected by an appropriate method which does not prejudice the viability of micro-organisms which may be present in the preparations to be tested. A pass-through hatch (or transfer box) is considered part of the testing environment. If the packages are double-wrapped and sterilised the outer wrapping should be removed just prior to the introduction of the package into the testing environment.

4. TEST METHODS

400. Relevant sections of the BP/Ph Eur: 'Test for sterility of the product to be examined'; 'Validation test'; 'Observation and interpretation of results'; 'Application of the test to parenteral preparations, ophthalmic and other non-injectable preparations required to comply with the test for sterility'

General methodology

401. Tests for sterility are carried out by the method of Membrane Filtration, by the method of Direct Transfer or by Addition of Concentrated Medium to the product. The method of Membrane Filtration should be used as the method of choice wherever feasible.

402. Fluid Thioglycollate Medium (Medium 1) and Soybean-Casein Digest Medium (Medium 2) are the media generally used for tests for sterility (see Section 6). Alternative media types may be appropriate where the nature of the product or method of manufacture can result in the presence of fastidious organisms (eg vaccines, blood products). Validation studies should indicate that alternative media are capable of supporting the growth of a wide range of micro-organisms in the presence of the product.

403. Where the preparation to be tested has antimicrobial effects, these effects may be reduced or neutralised by adding an appropriate substance to the specified test media, to diluents or solvents, or to the preparation prior to testing. Media so modified should be subjected to the tests described for unmodified media in clauses 608-616 and should only be used in tests for sterility if found to comply.

404. Containers of Medium 1 are incubated at 30 - 35°C and Medium 2 at 20 - 25°C.

Test method validation

405. Before tests for sterility for any product are initially carried out, it is necessary to demonstrate the validity of the test method used by recovery of a small number of micro-organisms in the presence of the product (see Annex I *Guidance on Obtaining Small Numbers of Vegetative Organisms and Spores*). It is preferable to add these challenge organisms directly to the product prior to membrane filtration or direct inoculation; where this is not practicable due to inhibition or irreversible binding by the product, the challenge organisms should be added to the last rinse solution if the membrane filtration method is used, or directly to the media containing the product if the direct method is used.

406. Validation should mimic the test proper in every detail, such as in the volumes of media used, quantities and dilutions of product and diluents: the approach depends on the method of test and details are given in each section. It may be performed concurrently with the actual test for sterility but should be confirmed as successful before the results of the sterility test are interpreted.

407. Validation is to be performed when the test for sterility has to be carried out on reformulated or new product, or whenever there is a change in the experimental conditions of the test. It is good practice to revalidate test methodology every 12 months although this is not a pharmacopoeial requirement and the frequency can be varied depending on frequency of manufacture, the nature and ingredients of the product and the frequency of stasis testing.

408. If a test method cannot be satisfactorily validated the regulatory authority should be notified.

409. All validation procedures should be carried out by personnel who are responsible for the routine testing of the product and should be done for each facility manufacturing that product.

Method of membrane filtration

Procedures

410. The filter should be a membrane filter disc of cellulose esters or other suitable plastics, having a nominal average pore diameter not exceeding 0.45µm. The membrane should be held firmly in a filtration unit which consists of a supporting base for the membrane, a receptacle for the fluid to be tested, a collecting reservoir for the filtered fluid, and the necessary tubes or connections. The apparatus is so designed that the solution to be filtered can be introduced and filtered under aseptic conditions. It permits the aseptic removal of the membrane for transfer to medium or it is suitable for carrying out the incubation after adding the medium to the apparatus itself.

411. Where the product has antimicrobial activity, the use of hydrophobic edged membranes is recommended so as to facilitate washing, unless self-contained canister systems are employed.

412. Cellulose nitrate filters are recommended for aqueous, oily and weakly alcoholic solutions and cellulose acetate filters for strongly alcoholic solutions.

413. The entire unit should be sterilised by appropriate means with the membrane filter and sterile airways in place. The method of sterilisation should not be deleterious to the membrane, eg, weaken it or change the nominal average pore diameter. The sterile airways should provide free access to the sterilising agent. After sterilisation, the apparatus should be free of leaks to the atmosphere except through the sterile airways.

414. The filter should be pre-wetted with diluent or solvent before filtration to minimise the retention of sample, particularly where small volumes and antibiotics are tested. A visual check of the integrity of the filter membrane should be carried out after filtration has been completed and the test shall be invalid if defects are apparent.

415. The specific diluents referred to in Table 4 and in the sections below are not obligatory and alternatives may be used provided they are compatible with the membrane and do not have antimicrobial activity as demonstrated by validation studies. It is assumed that membranes of approximately 50 mm diameter are used in the methods described below. If filters of a different diameter are used the volumes of diluents and wash solutions should be adjusted appropriately. The total volume washed through one single membrane should not exceed 1000 mL unless otherwise justified and authorised.

Aqueous solutions and suspensions which may be filtered directly

416. The prescribed volumes (Tables 2 and 3) of aqueous solutions and suspensions which can be filtered without prior treatment or dilution are transferred from the containers of the product to a sterile filtration unit. Filtration is then carried out with the aid of suction or pressure.

417. Without delay the filter membrane(s) should be washed not less than three times with the diluent specified in Table 4 or an equivalent. Throughout the operation the membrane should remain covered with liquid. If the original preparation contains a preservative or has inherent antimicrobial activity, additional washes may be needed and/or the diluent may include an antimicrobial inactivator. The volume of diluent must be equal to that used during validation.

418. After filtration and washing, aseptically divide the filter into two parts of approximately equal surface area and transfer one part to Medium 1 and the other part to Medium 2.

419. The volume of Medium 1 should be such that the air space above the medium in the container is minimised. The volume of Medium 2 should be such that sufficient air space is left above the medium to provide conditions that permit the growth of obligate aerobes. This condition applies irrespective of the filtration system used.

TABLE 4: SAMPLE TREATMENTS, SOLVENTS AND DILUENTS SUGGESTED FOR USE IN STERILITY TESTS USING THE METHOD OF MEMBRANE FILTRATION[1]

Class of Sample		**Treatment before filtration (solution or dilution)[3]**	**Diluent for washing after filtration[3]**
Aqueous solutions	Containing lecithin	-	Diluent 2
	Others	-	Diluent 1 or 2
	With ingredient containing beta-lactam ring		Diluent 3 (one or more of the washes)
Water soluble substances	Containing lecithin	Dissolve in sterile water or suitable solvent	Diluent 2
	Others	Dissolve in sterile water or suitable solvent	Diluent 1 or 2
	With ingredient containing beta-lactam ring	Dissolve in sterile water or suitable solvent or Diluent 3	Diluent 3 (one or more of the washes)
Water insoluble substances (in solid form or as aqueous suspension)	Containing lecithin	Suitable solvent	Diluent 2
	Not containing lecithin	Suitable solvent	Diluent 1 or 2
	Containing a beta-lactam antibiotic	Diluent 3 or other solvent containing penicillinase	Diluent 3
Ointments and oily preparations		Dissolve in suitable solvent[2]	Diluent 2

1. In every case an equally or more effective diluent may be substituted for that suggested in the table.
2. In some cases it may be necessary to use a mixture of solvents or several different solvents in succession. Where the sample contains an antibiotic with a beta-lactam ring it may be necessary to add penicillinase to one of the solvents or to the solvent mixture.
3. Refer to Annex III for composition and preparation of diluents.

420. If test apparatus is used in which medium is added to the apparatus and the membrane incubated *in situ,* the sample should be divided between two units or multiples thereof. Add Medium 1 to one of the units and Medium 2 to the other unit.

421. The minimum test is one in which a single membrane is divided into two parts and one part is inoculated into Medium 1 and one part into Medium 2. The number of containers tested may be increased to increase the statistical information provided by a test. If the full sample cannot be passed through a single membrane because of filtration difficulties or unacceptable levels of residual antimicrobial substance(s) in the filter membrane, the sample may be divided into portions and each filtered separately. However, the transfer of membranes or parts of membranes into the two media should not differ from the above proportions. Incubate test vessels of Medium 1 at 30 - 35°C and the vessels of Medium 2 at 20 - 25°C.

Aqueous solutions and suspensions which must be diluted or treated prior to filtration

422. Where aqueous solutions or suspensions must be diluted or treated prior to filtration they should be diluted with diluents specified in Table 4 or a suitable alternative sterile diluent or solvent and should be filtered, washed or otherwise treated as for those which may be filtered directly.

423. 'Suitable alternative' is any other sterilised diluent in which the suspended substance is soluble or which enables the substance to pass through the filter. Such alternative diluents should not exhibit antimicrobial activity as demonstrated by validation studies.

424. The diluted or treated preparation should be filtered as described above in clauses 416-421.

Soluble or dispersible solids

425. Prior to filtration, the quantity of a solid to be tested (see Tables 2 and 3), is transferred from each container to one or more vessels to be pooled, dissolved or otherwise treated as permitted by this method. This should be done in one or more vessels containing the suitable solvent.

426. In the case of solids in final dosage form, measured volumes of the suitable solvent may be added directly to the final containers, and the test sample may then be withdrawn in the form of a solution or a suspension.

427. Appropriate diluents for use in these operations are listed in Table 4. A 'suitable solvent' for dissolving a water-insoluble solid is a solvent or a liquid that facilitates dissolution of the solid and its passage through the filters. It should be sterile and should not exhibit antimicrobial activity or change the nominal pore diameter of the filter membrane in the conditions of the test as demonstrated by validation studies.

428. The resulting preparation should be filtered as described above in clauses 416-421.

Ointments and oily preparations

429. Oils and oily solutions of sufficiently low viscosity may be filtered without dilution through a dry membrane.

430. Viscous oils may be diluted as necessary with a suitable sterile diluent such as isopropyl myristate shown not to have antimicrobial activity in the conditions of the test.

431. Allow the oil to penetrate the membrane by its own weight, then filter, applying the pressure or suction gradually. Wash the membrane not less than three times by filtering through it about 100 mL of a suitable sterile solution such as Diluent 2.

432. Ointments in a fatty base and emulsions of the water-in-oil type may be diluted to 1 per cent in isopropyl myristate at a temperature of 40°C, but not more than 44°C. As rapidly as possible the preparation should be filtered and the membranes washed without delay, as described above for oils and oily solutions.

433. The resulting preparation and filters should be cultured as described above in clauses 418-421.

Table 5: Micro-organisms for use in growth promotion, validation and stasis tests[1]

Micro-organism		**Incubation Conditions**	
Species	Suitable strain	Temperature (°C)	Maximum duration
Type: anaerobic bacteria		30 – 35	3 days for growth promotion. 5 days for validation and stasis.
Clostridium sporogenes	ATCC 19404 CIP 79.3 NCTC 532 ATCC 11437		
Type: aerobic bacteria		30 – 35	3 days for growth promotion. 5 days for validation and stasis.
Staphylococcus aureus	ATCC 6538 CIP 4.83 NCTC 10788 NCIMB 9518		
Pseudomonas aeruginosa	ATCC 9027 NCIMB 8626 CIP 82.118		
Bacillus subtilis	ATCC 6633 CIP 52.62 NCIMB 8054	20 – 25	
Type: fungi		20 – 25	5 days
Candida albicans	ATCC 10231 IP 48.72 NCPF 3179		
Aspergillus niger	ATCC 16404 IP 1431.83 IMI 149007		

NOTE:

1. This Table incorporates Table 2.6.1.-1 (BP/Ph Eur) and Table 1 (USP).

Initial validation of the test method - testing for residual antimicrobial activity

434 To validate the test method, carry out the test procedures as described in the relevant section above, up to the final wash procedure. To the final wash add an inoculum of not more than 100 viable cells of each of the specified aerobic bacteria, anaerobic bacteria and fungi.. (For guidance on preparation of inocula, see Annex I *Guidance on Obtaining Small Numbers of Vegetative Organisms and Spores*).

435 Add *Clostridium sporogenes* ATCC 19404, *Staphylococcus aureus* ATCC 6538 and *Pseudomonas aeruginosa* ATCC 9027 to Medium 1 and *Candida albicans* ATCC 10231, *Bacillus subtilis* ATCC 6633 and *Aspergillus niger* ATCC 16404 to Medium 2. Other appropriate strains of challenge organisms are listed in Table 5..

436 After the final wash with the added micro-organisms has been passed through the filter, incubate one filter disc in Medium 1 at 30 - 35°C and one in Medium 2 at 20 - 25°C.

437 If different culture conditions are to be used in the test for justified reasons, these must be validated using challenge organisms appropriate for the conditions.

438. Periodically, strains of micro-organisms collected from the manufacturing environment should be used as challenge organisms.

439. Growth of each of the added micro-organisms should be apparent within 48 hours. If conspicuous growth does not occur within 5 days for each bacteria and fungi the test procedure is not valid and must be modified (e.g. by using additional washes, using antagonists to the antimicrobial agent or other procedure) until conspicuous growth does occur when tests as above are carried out.

440. If the membrane is found to be free of such antimicrobial activity when first tested or after modification of procedures, application of the test to every sample is not necessary. (See also clause 407).

Method of direct transfer

Procedures

Liquids and soluble or dispersible solids

441. Transfer the quantity of the preparation to be examined as indicated in Table 3 directly into Medium 1 and Medium 2. Approximately equal quantities of the preparation should be added to each vessel of medium. Incubate the test vessels of Medium 1 at 30 - 35°C and the vessels of Medium 2 at 20 - 25°C.

442. The volume of Medium 1 should be such that the air space above the medium in the container is minimised. The volume of Medium 2 should be such that sufficient air space is left above the medium to provide conditions that permit the growth of obligate aerobes.

443 Unless otherwise prescribed, in no case should the volume of material under test be greater than 10% of the volume of the medium alone, ie, 90% medium and 10% product.

444. In the case of soluble or dispersible solids, a measured volume of Purified Water, or a suitable sterilised diluent or solvent which does not manifest antimicrobial activity as demonstrated by validation studies, should be added to each container of the solid. After the contents have been dissolved or dispersed, the specified quantity of the product should then be added in the form of a solution or suspension to the test media. Alternatively, the solid material may be transferred directly to the test media.

445. If a large volume of product is to be tested it may be preferable to use concentrated media, prepared so as to take the subsequent dilution into account. Where appropriate the concentrated medium may be added directly to the product in its container.

446. Any additional diluents, solvents or procedures for carrying out the test should be validated.

Solid articles

447. Wherever possible solid articles such as devices should be tested by immersion in or filling with culture media.

448. Aseptically dismantle all articles as completely as possible. Articles such as tubing may need to be cut. Other articles may need to be broken into smaller parts to allow access of medium to all surfaces of the article.

449. Immerse all parts of each article in sufficient medium contained in one vessel to completely cover all parts. The volume of Medium 1 should be such that the air space above the medium in the container is minimised. The volume of Medium 2 should be such that sufficient air space is left above the medium to provide conditions that permit the growth of obligate aerobes.

450. Place half the articles into Medium 1 and the remaining half into Medium 2. Incubate the test vessels of Medium 1 at 30 - 35°C and the vessels of Medium 2 at 20 - 25°C.

451. Where the product is a dressing, the whole article need not be tested. As a minimum, portions of 100-500 mg should be cut from that part of the dressing that is most inaccessible to sterilant. Articles may be pooled.

452. If the size of an article is such that all its parts are not covered by 2000 mL of medium, then those parts likely to be most easily accessible to the sterilant may be omitted. Unless the product is a dressing, before a test is adopted which omits parts of an article from routine testing, the proposed procedure should be discussed with the competent authority. Parts of an article should not be omitted from testing in order for articles to be pooled.

453. Alternatively, a larger vessel containing additional medium and capable of accommodating all parts of the article may be used.

454. Alternatively, if a large article cannot readily be cut into pieces, or only the fluid pathway of the device is intended to be sterile, medium should be added to the article aseptically and it should then be sealed and incubated.

455. If none of the above methods is practicable the article may be rinsed three times with suitable volumes of medium, so that all surfaces of the article which are required to be sterile come into intimate contact with medium. The entire washings from each article are then tested by the method of Membrane Filtration. This method is not as sensitive as those described above because micro-organisms adhering to surfaces may not be removed by washing. It should be used only as a last resort.

456. Care should be taken to ensure that entrapped air does not prevent the medium from making contact with all parts of the internal surfaces of an article. To facilitate this contact a surfactant agent is included in Medium 1 and Medium 2; Medium 1 may also be modified by the omission of agar.

Ointments and oily preparations

457. Ointments and oily preparations may be tested by the method of Direct Transfer if testing by the method of Membrane Filtration is not feasible, i.e. when a suitable solvent is not available (see clauses 429-430).

458. Before addition to media, ointments and creams may be diluted approximately 1 in 10 by emulsifying with a suitable emulsifying agent in a suitable sterile diluent to improve contact between the sample and the medium (polysorbate 80 or light liquid paraffin may be useful). In this case it may be appropriate to use Medium 1 and Medium 2 without polysorbate 80.

459. For oily liquids media containing an emulsifying agent should be used. Polysorbate 80 at 10 g/L, (p-tert-octylphenoxy) polyoxyethanol at 1 g/L, or other emulsifying agents in appropriate concentration may be suitable.

Initial validation of the test method - testing for antimicrobial activity

460. The goods to be tested for sterility should be tested for antimicrobial activity during the product development stages, if this is possible. If they are found to have such activity, preparatory or test procedures will need to be modified to neutralise this activity.

461. If goods are found to be free of such activity when first tested, or after modification of procedures, application of the test for antimicrobial activity to every sample is not necessary. (See also clauses 405-409).

462 To demonstrate that the mixture does not manifest antimicrobial activity carry out the test as described above up to the incubation step and add an inoculum of viable cells of the specified aerobic bacteria, anaerobic bacteria and fungi.

463 To one vessel containing the test sample in Medium 1, add an inoculum of *Clostridium sporogenes* ATCC 19404, *Staphylococcus aureus* ATCC 6538 and *Pseudomonas aeruginosa* ATCC 9027 and incubate at 30 – 35°C. To a second vessel containing the test sample in Medium 2 add an inoculum of *Candida albicans* ATCC 10231, *Bacillus subtilis* ATCC 6633 and *Aspergillus niger* ATCC 16404 to Medium 2 and incubate the vessel at 20 – 25°C.

464 Other appropriate strains of challenge organisms are listed in Table 5.

465 In each case the number of micro-organisms in the inoculum is to be not more than 100 CFU. (For guidance on preparation of inocula, see Annex I Guidance on Obtaining Small Numbers of Vegetative Organisms and Spores).

466. Growth of each of the added micro-organisms should be apparent within 48 hours. If conspicuous growth does not occur within 5 days, the test procedure is not valid and must be modified (e.g. by using additional washes, using antagonists to the antimicrobial agent or other procedure) until conspicuous growth does occur when tests as above are carried out.

467. Periodically, the strains referred to above should be supplemented by strains of micro-organisms collected from the manufacturing environment.

Negative product control tests

468. The results of negative product control tests facilitate the interpretation of sterility test results, particularly when used to declare a test invalid because of contamination in the negative product controls.

469. During each working session (i.e. that uninterrupted period of time in which a sample or group of samples is tested) in which sterility testing is carried out, at least ten negative product control containers should be tested. For a direct inoculation test these controls should be tested where possible at regular intervals during the test session.

470. A negative product control is usually a terminally sterilised item of undoubted sterility, that is, it has been subjected to the equivalent of two sterilisation cycles by autoclaving or by dry heat sterilisation, or 50 kGy of gamma irradiation. Acceptable alternatives could be a container that has been aseptically filled and then subjected to 25 kGy or a container of medium that has been filled during a media fill validation, incubated for 14 days and been found to be sterile.

471. A negative control should be similar in type and container (or packaging if a device) to the product under test. The essential element of the negative control is that the manipulations involved in testing the control should be similar to those involved in testing the product. There should be similar risks of introducing contamination in the control and product tests.

472. A suitable negative product control for an aqueous product could be distilled water in a similar container. A negative product control for testing an ointment could be a container of liquid paraffin or ointment base that has been sterilised by dry heat; pouring the liquid paraffin from a container would be adequate to simulate squeezing of ointment from a tube. For disposable devices, a section of glass or plastic tubing packaged in a manner similar to the device, and sterilised by gamma irradiation, could serve as a negative control.

473. Where a retest is being carried out in the working session these simulated negative controls should be processed concurrently with that retest.

474. The negative control contamination rate should be calculated and recorded. In order to derive the maximum information from the results of sterility tests it is essential that the level of contamination detected in negative control tests be minimal.

Incubation and examination of sterility tests

475. Incubate all test vessels of Medium 1 (or equivalent medium - see clause 402) at 30 - 35°C. Incubate the vessels of Medium 2 (or equivalent medium - see clause 402) at 20 - 25°C.

476. All vessels should bear the identity of the product or control being tested, the medium used, the temperature of incubation and date of inoculation.

477. All test and control vessels, other than the subcultured vessels referred to below, must be incubated for at least 14 days unless microbial contamination is detected at an earlier time.

478. At intervals during the incubation period examine each vessel for evidence of microbial growth; a suitable interval is 2 working days. Care should be taken to prevent undue agitation of the Medium 1. At the end of the incubation period examine each vessel again for evidence of microbial growth after agitating, swirling or inverting the contents.

479. Preparations that produce a suspension, flocculation or deposit so that the presence or absence of microbial growth cannot be readily seen should be mixed by gentle swirling or inversion at each examination until subcultured. Care should be taken to prevent undue agitation of the Medium 1 and to ensure that anaerobic conditions are maintained as indicated by the resazurin indicator. After 14 days incubation transfer a suitable portion (not less than 1 mL) of the contents to a fresh vessel of the same medium. Incubate the subcultured vessels for not less than 4 days at the same temperature as that at which the original vessel was incubated. Continue incubation of the original and the subcultured vessels for a total of not less than 14 + 4 days from the original inoculation.

480. If turbidity, precipitate, or other evidence of microbial growth during incubation is seen:
- examine the suspected growth microscopically by Gram stain;
- attempt to grow single colonies using appropriate microbiological methods;
- examine colonies of each type of micro-organism present for their colonial morphology and cellular morphology by Gram stain;
- attempt to identify the isolates, as far as the genus, and preferably species.

NOTE: If the identity of isolates is to be used as the basis for invalidating a test, a sensitive method of identification such as molecular typing techniques using RNA/DNA homology is required (see also clause 495).

481. Keep records of these cultures in order to detect a pattern of recurring contaminants in the product. It is recommended that cultures of recurring contaminants be maintained in pure form and used as reference organisms for evaluation of environmental background contamination.

482. Automated or semi-automated biochemical organism identification systems should be subjected to periodic verification using reference strains of organisms that can be traced to a recognised reference culture collection, such as the American Type Culture Collection (ATCC), Maryland, USA, or the National Collection of Type Cultures (NCTC), London, UK.

Monitoring the efficacy of test media at the end of the incubation period (stasis test)

483. The stasis test is not mandated by the Pharmacopoeias but is recommended as part of Good Laboratory Practice (GLP) in relation to method validation and a quality system based on ISO 17025. It is particularly important for antibiotics, slow-release sterile products and for direct inoculation methods where validity of the test depends on the use of an exact amount of product (ie, marginal methodology).

484. The stasis test is intended to demonstrate that the media inoculated with the test preparation will support growth for the full incubation period. For example, it is necessary to show that anaerobiosis is maintained in the Medium 1 to allow the late development of slow-growing anaerobes. It is also necessary to demonstrate that growth promoting qualities of media are retained and that preservative inhibitors remain stable for the full test period.

485. The stasis test will be included in referee testing (see clauses 101 and 105).

486 After incubation of the media has been completed in accordance with the instructions given in clauses 475-479:

- add to representative vessels containing Medium 1 that has been incubated at 30 – 35°C, an inoculum of viable spores of an anaerobic bacterium eg, *Clostridium sporogenes* ATCC 19404;
- add to representative vessels containing Medium 2 that has been incubated at 20 – 25°C an inoculum of viable cells of a fungus, eg, *Candida albicans* ATCC 10231;

NOTE: Acceptable challenge organisms are listed in Table 5. The list is not exclusive; other micro-organisms may be suitable.

487. In each case the number of organisms in the inoculum is to be not more than 100 CFU (see Annex I *Guidance on Obtaining Small Numbers of Vegetative Organisms and Spores*).

488. The vessels are returned to their previous temperature and incubation continued. The containers of product should all show growth of the added organisms within 48 hours. If conspicuous growth is not apparent within 5 days for both bacteria and fungi the test is considered invalid. Invalid stasis tests may be repeated once. If conspicuous growth is not obtained at the second attempt the test method should be modified and revalidated.

489. If the media are found to support growth of the test micro-organisms then this test need not be applied to every sample. It should be repeated periodically on the relevant categories of products or when product is reformulated. Every 12 months is recommended.

490. Periodically the strains referred to above may be supplemented with appropriate strains of micro-organisms collected from the manufacturing environment.

Interpretation of the test results

491. If microbial growth is not evident in any of the vessels inoculated with the product, the sample tested complies with the test for sterility, provided that growth of challenge organisms has been demonstrated in the stasis test (if performed), in growth promotion tests on the batches of media used and in test method validation. This interpretation applies even if growth occurs in negative product control vessels.

492. If microbial growth is evident the product does not comply with the test for sterility unless it can be clearly demonstrated that the test was invalid for causes unrelated to the product being examined.

493. If microbial growth is evident, the criteria for invalidating the test are:
(a) the data of the microbiological monitoring of the sterility testing facility show a fault;
(b) a review of the testing procedure used during the test in question reveals a fault;
(c) microbial growth is found in the negative product controls;

(d) after determination of the identity of the micro-organisms isolated from the test, the growth of this species or these species may be ascribed unequivocally to faults with respect to the material and/or technique used in conducting the sterility test procedure.

494. When conditions (a), (b) or (c) apply, the test should be aborted prior to the completion of the incubation period.

495. If condition (d) is to be used as the sole criterion for invalidating a sterility test, it is necessary to employ sensitive typing techniques to demonstrate that a microorganism isolated from the product test is identical to a microorganism isolated from the materials and/or the environment. While routine biochemical/phenotypical identification techniques can demonstrate that two isolates are not identical, these methods are not sufficiently sensitive or reliable enough to provide unequivocal evidence that two isolates are from the same source. Suitably sensitive tests (for example, molecular typing with RNA/DNA homology) are those accepted by microbiologists conducting epidemiological studies to determine that microorganisms are clonally related and have a common origin. Repeat testing based on the biochemical or phenotypical characterisation of environmental and/or product isolates should not be permitted. The test environment can be contaminated by actual product samples, which may contain multiple micro-organisms that are difficult to speciate without employing sensitive typing techniques.

496. If the test is declared to be invalid it may be repeated with the same number of units as in the original test.

497. If there is no evidence of growth in any vessels inoculated with the product during the repeat test the product passes the test for sterility. This interpretation applies even if growth occurs in negative product control vessels.

498. If there is evidence of growth in the test vessels the product fails the test for sterility. Further testing is not permitted under any circumstances.

499. If two consecutive tests on the same product give evidence of growth in control vessels, or consecutive working sessions give evidence of growth in controls, or there is any other evidence of breakdown in testing methods, then there should be a complete review of all facilities and testing procedures to determine the cause of the contamination. Further tests on samples should be suspended until the review is completed.

5. RECORDS

500. Reference: PIC/S *Recommendations on Sterility Testing (PS/W 2/98)*.

501. Records should be kept of all sterility testing which is carried out.

502. For each test these records should contain at least the following information:
- description and number of product units tested;
- batch/lot number;
- stage of manufacture (finished product, intermediate or final bulk);
- personnel performing tests;
- dates of testing;
- test methodology (volume tested, diluents/solvents used, media, media batch numbers, temperature and time of incubation);
- results in full.

503. Records should also be maintained of:
- details of validation of the sterility test method;
- periodic stasis testing;
- details of any product contamination irrespective of whether the test was valid or invalid;
- the negative control contamination rate;
- results of environmental and personnel monitoring.

504. Results of sterility testing for test samples and negative controls should be presented in a format that allows for easy recognition of trends.

505. These records should be appropriately stored and readily available as defined in Chapter 4 of the *Australian Code of Good Manufacturing Practice for Medicinal Products*, *2002*.

6. MEDIA FOR USE IN STERILITY TESTING

600. Relevant section of the BP/Ph Eur**: 'Culture Media and Incubation Temperatures'**

Composition

601. Fluid Thioglycollate Medium (Medium 1) and Soybean-Casein Digest Medium (Medium 2) are the media generally used for tests for sterility. The composition of these media should be as specified in Annex II. Commercially available dried media, which differ slightly from the specified composition, may be used provided the reconstituted medium has been shown to support the growth of aerobic and anaerobic bacteria and fungi, as described in clauses 611-615.

602. As noted in clause 402, alternative media types may be appropriate where the nature of the product or method of manufacture may result in the presence of fastidious organisms (eg, vaccines, blood products).

603. Inactivators of antimicrobials may be incorporated into culture media or solutions if indicated by validation studies.

604. Media may be either purchased from an approved supplier or manufactured in-house.

Method of preparation

605. Media should be prepared according to written procedures that are based on a validated sterilisation process.

606. Every batch should be assigned a unique batch number and expiry date and its manufacture documented.

607. Detailed guidance for preparation of Medium 1 and Medium 2 is provided in Annex II.

Tests on the media

608. Before use, each batch of the sterilised media should be tested for pH, sterility and growth promotion.

609. Measure the pH of a sample of each batch of medium. Any batch of medium of pH not within the range prescribed in Annex II for that medium should not be used in tests for sterility.

610. To check for sterility, incubate the media at 30 - 35°C and 20 - 25°C for 14 days. This testing may be performed on 100% of the batch or on representative portions and may be conducted concurrently with the product sterility test. Media which contain visible particulate matter should not be used in tests for sterility. Parametric release of media may be permitted if media is manufactured under the same conditions and controls as products already approved by the competent authority for parametric release (see also clause 708).

611. The ability of media to support the growth of micro-organisms should be tested by addition of small numbers of challenge organisms (see Annex I *Guidance on Obtaining Small Numbers of Vegetative Organisms and Spores*). After each batch of medium has been sterilised at least two vessels are selected from positions in the steriliser load where the exposure to heat is likely to be maximal.

612. Where the medium under examination is Medium 1 add not more than 100 viable spores/CFU of each of the following species of micro-organisms: *Clostridium sporogenes* ATCC 19404, *Staphylococcus aureus* ATCC 6538 and *Pseudomonas aeruginosa* ATCC 9027.

613. Where the medium under examination is Medium 2 add not more than 100 viable cells of *Candida albicans* ATCC 10231, *Bacillus subtilis* ATCC 6633 and *Aspergillus niger* ATCC 16404 to selected vessels.

614. Other appropriate strains of challenge organisms are listed in Table 5. It is recommended that from time to time the routine strains should be supplemented by using strains collected from the manufacturing environment.

615. Growth of the challenge micro-organisms should be apparent within 48 hours. If growth is not conspicuous in each of the incubated vessels within 3 days in the case of bacteria or 5 days in the case of fungi, the batch should not be used in tests for sterility.

616. Prepared media purchased from external vendors should be accompanied by certification of the growth promotion test performed on each batch of media. The test need not be performed by the sterility testing laboratory provided there is documented control over the conditions used to transport media between the media manufacturer and the testing laboratory.

Shelf-life

617. Media may be stored at 2 – 25°C in suitable sealed containers but must not be used after storage periods that have not been validated. It should be tested for growth promotion every three months.

618. Medium 1 of which more than the upper one-half is pink in colour should not be used in tests for sterility. Vessels of this medium which have become excessively pink may be heated once only in a steam bath, or in freely flowing steam, until the pink colour disappears.

7. DILUENTS, SOLVENTS AND WASH SOLUTIONS FOR USE IN STERILITY TESTING

700. Relevant sections of the BP/Ph Eur: 'Test for sterility of the product to be examined'; 'Application of the test to parenteral preparations, ophthalmic and other non-injectable preparations required to comply with the test for sterility'.

Composition

701. The composition of the diluents referred to in Table 4 (Diluents 1, 2 and 3) are specified in Annex III.

702. These diluents may be modified by addition of antimicrobial inactivators or other equally or more effective diluents may be used, if indicated by validation studies.

Method of preparation

703. Diluents, solvents and wash solutions should be prepared according to written procedures that are based on a validated sterilisation process.

704. Every batch should be assigned a unique batch number and expiry date and its manufacture documented.

705. Detailed guidance for preparation of the diluents listed in Table 4 is provided in Annex III.

Tests on diluents, solvents and wash solutions

706. Diluents, solvents and wash solutions should be tested before use for pH and where practicable, for sterility.

707. Measure the pH of a sample of each batch. Any batch whose pH is not within the range specified in Annex III for that diluent should not be used in tests for sterility.

708. To check for sterility, incubate the preparation at 30 - 35°C and 20 - 25°C for 14 days. This testing may be performed on 100% of the batch or on representative portions and may be conducted concurrently with the product sterility test. Solutions that are turbid, or which contain visible particulate matter, should not be used in tests for sterility. Parametric release of diluents and solvents may be permitted by the competent authority (see also clause 610).

Shelf-life

709. Diluent 3 which is more than 10 days old should not be used in tests for sterility.

ANNEX I. GUIDANCE ON OBTAINING SMALL NUMBERS OF VEGETATIVE ORGANISMS AND SPORES

All procedures for the preparation, maintenance and cultivation of challenge organisms should be documented. At the time of use, cultures maintained by seed lot culture techniques should be no more than 5 passages from the original type culture strain that has been obtained from a recognised reference culture supplier. The identity (morphological and physiological properties) should be checked periodically.

The methods described below are for preparing suspensions of low numbers of the challenge organisms from the major groups (aerobes, anaerobes and fungi) in Table 5. They are provided as examples of acceptable procedures and are for guidance only. Some incubation times and temperatures differ from those in the sterility test procedures; they are suggested because they have been found to provide, for the particular strains used, conditions under which useful numbers of viable cells can be cultured within 24 hours.

The methods detailed may be modified for other organisms by changing the media, diluents or culture conditions. Other methodology (such as the use of suspensions on glass beads or suspensions stored in a 10% glycerol preparation at -70°C) may be equally satisfactory. Irrespective of the method used, it is advisable for any laboratory to carry out viable counts on the selected working dilution of each challenge organism on a daily basis for a whole week to determine the viability (stability) of their particular strains of organisms.

Method for preparation of cell suspension of *Staphylococcus aureus* or *Pseudomonas aeruginosa*

Every 4 months open a new ampoule and subculture to Soy Bean Casein Digest (SCD) broth. The incubation conditions for these organisms are 24 hours at 37°C for *S. aureus* and 30°C for *P. aeruginosa*. Subculture from the SCD broth to Soy Bean Casein Digest agar (SCDA) slopes (stock slopes) and concurrently plate on to a SCDA plate to check for purity.

Every month subculture from the stock slope to a fresh SCDA slope.

Every week subculture from the monthly slope to a SCDA plate. If the culture is pure, subculture from the slope into 10 mL of SCD and incubate for 24 hours at 37°C for *S. aureus* and 30°C for *P. aeruginosa*.

This culture is used to prepare the working dilution as follows:

- assuming that the 24 hour culture contains 1 x 10^9 CFU/mL, carry out sufficient serial dilutions in 0.1% peptone saline to arrive at approximately 100 CFU/mL;
- prepare about 100 mL of this dilution, which will be the working dilution.

Carry out a viable count on the working dilution on SCDA plates.

From the results of the viable count calculate the volume of the working dilution which contains not more than 100 CFU and use this for validation, growth promotion and stasis testing. A concurrent viable count should be carried out when performing any of these tests, as a check that the working dilution has been correctly prepared and calculated.

Store the 100 mL of the working dilution at 2-8°C. Use as needed but do not keep longer than a week.

Method for Preparation of Cell Suspension of *Candida albicans*

Every 4 months open a new ampoule and subculture to Sabouraud Dextrose Broth (SDB). Unless otherwise stated the incubation conditions for this organism are 24-48 hours at 30°C. Subculture from the SDB to Sabouraud Dextrose Agar (SDA) slopes (stock slopes) and concurrently plate on to an SDA plate to check for purity. Prepare sufficient stock slopes to last 4 months.

Every month subculture from the stock slope to a fresh SDA slope.
Every week subculture from the monthly slope to a SDA plate. If the culture is pure, subculture from the slope into 10 mL of SCD and incubate for 24 hours at 30°C.
This culture is used to prepare the working dilution, as follows:

- assuming that the 24 hour culture contains 1 x 10^8 CFU/mL, carry out sufficient serial dilutions in 0.1% peptone saline to arrive at approximately 100 CFU/mL;
- prepare about 100 mL of this dilution, which will be the working dilution.

Carry out a viable count on the working dilution on SDA plates and incubate at 30°C for 24 hours.

From the results of the viable count calculate the volume of the working dilution that contains not more than 100 CFU and use this for validation, growth promotion and stasis testing. A concurrent viable count should be carried out when performing any of these tests, as a check that the working dilution has been correctly prepared and calculated.

Store the 100 mL of the working dilution at 2-8°C. Use as needed but do not keep longer than a week.

Preparation of spore suspensions of *Bacillus subtilis*

Stock suspension

Preparation of the stock suspension may be carried out every 12 months.

Open ampoule and subculture into SCD and incubate at 37°C for 24 hours.

Inoculate five 45 mL Sporulation Agar slopes (in 100 mL medical flats) with approximately 1.0 mL of the 24 hour broth culture and incubate at 37°C for 5 days. Concurrently, plate the 24 hour broth culture onto SCDA to check for purity. Incubate at 37°C overnight. Next day, if pure, discard; otherwise purify.

Check spore production after 5 days by spore stain. If the percentage of cells sporing is less than 70-80% continue incubation. When a 70-80% spore yield is achieved, wash off the growth from the flats with 20 mL of sterile normal saline and dispense into sterile McCartney bottles or centrifuge tubes.

Centrifuge at 1500 rpm for 20 minutes. Decant (and discard) the supernatant liquid.

Resuspend the sediment in 10 mL of fresh sterile normal saline and spin again; repeat this process three times.

After the third wash decant off the supernatant liquid except for approximately 1mL. Resuspend the spores in 2 mL of normal saline.

Heat the spore suspension at 56°C for 30 minutes to kill the vegetative cells.

Carry out a viable count on SCDA using peptone saline as diluent, incubating at 37°C for 24 hours. The final preparation should contain approximately 10^8 spores/mL.

Working dilution suspension

To prepare a working dilution, dilute the spore suspension in peptone saline to contain approximately 100 spores/mL. Prepare sufficient to last a week for validation, growth promotion and stasis testing. Keep refrigerated at 2-8°C.

Carry out a viable count on the working dilution on SCDA plates; incubate the plates at 37°C for 24 hours.

From the results of the viable count calculate the volume of the working dilution that contains not more than 100 CFU and use this for validation, growth promotion and stasis testing. A

concurrent viable count should be carried out when performing any of these tests, as a check that the working dilution has been correctly prepared and calculated.

Preparation of spore suspension of *Clostridium sporogenes*

Stock suspension

Preparation of the stock suspension may be carried out every 12 months.
Open ampoule and inoculate into Reinforced Clostridial Medium (RCM). Incubate under anaerobic conditions at 32°C for 48 hours.

Subculture about 3-5 mL of the broth onto each of five 45 mL solid RCM agar slopes (in 100 mL medical flats) and incubate anaerobically at 32°C for until 70-80% of the population is sporing (approximately 2 weeks). Spore production should be checked every few days by spore stain.

When a 70-80% spore yield is achieved, wash off the growth from the flats with 20 mL of sterile normal saline and dispense into sterile McCartney bottles or centrifuge tubes.

Centrifuge at 1500 rpm for 20 minutes. Decant (and discard) the supernatant liquid.

Resuspend the sediment in 10 mL of fresh sterile normal saline and spin again; repeat this process three times.

After the third wash decant off the supernatant liquid except for approximately 1mL. Resuspend the spores in 2 mL of normal saline.

Heat the spore suspension at 56°C for 30 minutes to kill the vegetative cells.

Carry out a viable count on SCDA using peptone saline as diluent, incubating anaerobically at 32-37°C for 24-48 hours. The final preparation should contain approximately 10^8 spores/mL.

Working dilution suspension

To prepare a working dilution, dilute the spore suspension in peptone saline to contain approximately 100 spores/mL. Prepare sufficient to last a week for validation, growth promotion and stasis testing. Keep refrigerated at 2-8°C.

Carry out a viable count on the working dilution on SCDA plates; incubate the plates anaerobically at 32-37°C for 24-48 hours.

From the results of the viable count calculate the volume of the working dilution that contains not more than 100 CFU and use this for validation, growth promotion and stasis testing. A concurrent viable count should be carried out when performing any of these tests, as a check that the working dilution has been correctly prepared and calculated.

Preparation of spore suspensions of *Aspergillus niger*

Stock suspension

Preparation of the stock suspension may be carried out every 12 months.

Open ampoule and subculture into SDB and incubate at 25°C for 3 days.

Inoculate five 45 mL SDA slopes (in 100 mL medical flats) with approximately 1.0 mL of the broth culture and incubate at 25°C for 5 days. Concurrently, plate the broth culture onto SDA to check for purity. Incubate at 25°C for 3 days. Next day, if pure, discard; otherwise purify.

Check spore production after 5 days by the appearance of black spores on the surface. When the entire surface of the culture is black, wash off the growth from the flats with 20 mL of sterile normal saline and dispense into sterile McCartney bottles or centrifuge tubes.

Centrifuge at 1500 rpm for 20 minutes. Decant (and discard) the supernatant liquid.

Resuspend the sediment in 10 mL of fresh sterile normal saline and spin again; repeat this process three times.

After the third wash decant off the supernatant liquid except for approximately 1mL. Resuspend the spores in 2 mL of normal saline.

Carry out a viable count on SDA using peptone saline as diluent, incubating at 25°C for 3 days. The final preparation should contain approximately 10^8 spores/mL.

Working dilution suspension

To prepare a working dilution, dilute the spore suspension in peptone saline to contain approximately 100 spores/mL. Prepare sufficient to last a week for validation, growth promotion and stasis testing. Keep refrigerated at 2-8°C.

Carry out a viable count on the working dilution on SDA plates; incubate the plates at 25°C for 3 days.

From the results of the viable count calculate the volume of the working dilution that contains not more than 100 CFU and use this for validation, growth promotion and stasis testing. A concurrent viable count should be carried out when performing any of these tests, as a check that the working dilution has been correctly prepared and calculated.

ANNEX II. COMPOSITION AND PREPARATION OF MEDIA

Relevant section of the BP/Ph Eur: 'Culture media and incubation temperatures'

This Annex describes methods for preparation and sterilisation of the standard sterility test media (see also Section 6). Commercially available dried media that differ slightly from the specified composition may be used provided the reconstituted medium has been shown to support the growth of aerobic and anaerobic bacteria and fungi, as described in clause 611-615. Alternative media types may be appropriate where the nature of the product or method of manufacture may result in the presence of fastidious organisms (eg, vaccines, blood products).

Inactivators of antimicrobials may be incorporated into culture media or solutions if indicated by validation studies.

If heat labile additives such as serum are included the media may be sterilised by a validated filtration method. Cogent reasons would be required to justify the use of filtration as a method of sterilisation of media that can be terminally sterilised.

Medium 1 (Fluid Thioglycollate Medium)

Composition

Pancreatic Digest of Casein	15.0 g
Yeast Extract (water-soluble)	5.0 g
Glucose monohydrate/anhydrous	5.5 g/5.0 g
Sodium chloride	2.5 g
L-Cystine	0.5 g
Sodium thioglycollate	0.5 g
0.1% Resazurin Sodium Solution (freshly prepared)	1.0 mL
Granulated Agar (moisture not more than 15%)	0.75 g
Purified Water	1000 mL
Polysorbate 80 (optional)	5.0 mL

pH after sterilisation (measured at room temperature): 7.1± 0.2

Method of preparation

Either

Mix the pancreatic digest of casein, yeast extract, glucose, sodium chloride, L-cystine, agar and water in the proportions specified above and heat until dissolved. Dissolve the sodium thioglycollate in the solution. Add the specified quantity of Polysorbate 80 if this ingredient is to be included. If necessary, add sufficient 1 M sodium hydroxide or 1 M hydrochloric acid so that after the solution is sterilised its pH will be 7.1± 0.2. If the solution is not clear, heat to boiling but do not boil, and filter while hot through moistened filter paper. Add the resazurin sodium solution and mix.

Or

Dissolve a mixture of dehydrated mixture ingredients in the specified proportions, in water. Observe the instructions provided by the manufacturer of the dehydrated mixture to effect solution and to obtain a clear solution of the specified pH. Immediately prior to adjusting the pH, add the specified quantity of Polysorbate 80 if this ingredient is to be included.

Medium 2 (Soybean-Casein Digest Medium)

Composition

Pancreatic Digest of Casein	17.0 g
Papain Digest of Soybean Meal	3.0 g
Glucose monohydrate/anhydrous	2.5 g/2.3 g
Sodium chloride	5.0 g
Dipotassium hydrogen phosphate, K_2HPO_4	2.5 g
Purified Water	1000 mL
Polysorbate 80 (optional)	5.0 mL

pH after sterilisation (measured at room temperature): 7.3±0.2

Method of preparation

Either

Mix the ingredients, in the proportions specified above, warming slightly to effect solution. Cool the solution to room temperature. Add the specified quantity of Polysorbate 80 if this ingredient is to be included. If necessary, add sufficient 1 M sodium hydroxide or 1M hydrochloric acid so that after the solution is sterilised its pH will be 7.3± 0.2. If the solution is not clear filter through moistened filter paper.

Or

Dissolve a mixture of dehydrated ingredients in water in the amount necessary to obtain the required concentration. Follow the instructions provided by the manufacturer to obtain a clear solution of the specified pH. Just prior to adjusting the pH, add the specified quantity of Polysorbate 80 if this ingredient is to be included.

Both media

Filling and containers

Distribute all media into clear colourless glass vessels with external screw threaded necks in the required volumes. Each vessel for Medium 1 should provide a ratio of surface to depth of medium such that when inoculated with a sterile inoculum not more than the upper one-half of the medium becomes pink in colour at the conclusion of the test for sterility. The capacity of each vessel for Medium 2 should be at least twice the volume of the medium placed in it. Close all vessels with aluminium or heat resistant plastic screw-caps.

Sterilisation

Media should be sterilised within four hours of the start of its preparation.

Sterilise the vessels of media by exposing them to saturated steam in an autoclave for a time sufficient to ensure that the whole of the contents of each vessel is maintained at 121°C for 20 minutes. In the process of sterilisation do not prolong the heating up or delay the cooling down of the media unnecessarily. Bring the pressure in the autoclave chamber to atmospheric pressure at such a rate so as not to cause the medium to boil over. Allow the media to cool to about 30°C, either in the autoclave chamber or a sterile environment, then tighten the cap of each vessel. Cooling may be carried out in a controlled clean environment provided that the process has been validated.

Alternative validated terminal sterilisation methods may be used.

ANNEX III. COMPOSITION AND PREPARATION OF DILUENTS

Diluent 1

Composition

Peptic Digest of Animal Tissue	1 g
Purified Water	1000 mL
pH after sterilisation (measured at room temperature):	7.1±0.2

Method of preparation

Mix the ingredients in the proportions specified above and heat to dissolve. If necessary, add sufficient
1 M sodium hydroxide or 1 M hydrochloric acid to bring the pH within the range specified for the diluent. Distribute into vessels that are closed against the access of micro-organisms, and sterilise the filled vessels by heating in an autoclave by the method described for sterilisation of media.

Sterilisation

If necessary, clarify the diluents by filtration, preferably through membrane filters, before sterilisation. Sterilisation should be performed within four hours of the start of preparation and should be by exposure of the whole of the contents of each vessel to 121°C for 20 minutes.

Diluent 2

Composition

Peptic Digest of Animal Tissue	1 g
Polysorbate 80	1 mL
Purified Water	1000 mL
pH after sterilisation (measured at room temperature):	7.1± 0.2

Method of preparation and sterilisation

Prepare and sterilise as above for Diluent 1.

Diluent 3

Composition

Peptic Digest of Animal Tissue	1 g
Disodium hydrogen phosphate, Na_2HPO_4	8.38 g
Sodium dihydrogen phosphate, $NaH_2PO_4.2H_20$	1.62 g
Purified Water	1000 mL
Penicillinase solution (15000 Levy Units/mL)	100 mL
Final pH (measured at room temperature):	7.5±0.2

Method of preparation

Proceed as in the preparation of Diluents 1 and 2. After the sterilised solution has cooled to between 20°C and 30°C, aseptically add the penicillinase.

ANNEX IV. STERILITY TEST ENVIRONMENT

This Annex provides guidance for achieving a testing environment that is equivalent to the conditions required for the aseptic manufacture of pharmaceutical products, as required by the BP/Ph Eur (section 'Precautions against microbial contamination'). It incorporates relevant elements from the Pharmaceutical Inspection Convention/ Pharmaceutical Inspection Co-operation Scheme (PIC/S) *Recommendations on Sterility Testing* (April 2000), Annex 1 of the PIC/S *Guide to Good Manufacturing Practice for Medicinal Products* (August 2001) and the *Australian Code of Good Manufacturing Practice for Medicinal Products Annex 1 – Manufacture of Sterile Medicinal Products.*

Clean-rooms

Classification

Sterility testing should be performed under conditions that meet Class 3.5 of Australian Standard 1386 or the equivalent European Class A (see Table 6).

The BP/Ph Eur requires that sterility testing is carried out 'under aseptic conditions'. Guidance is provided that these conditions can be achieved using, for example, a class A laminar-air-flow cabinet located within a class B clean-room or an isolator' (BP/Ph Eur: 'Precautions Against Microbial Contamination').

A Class 3.5 laminar flow cabinet stationed within an AS Class 350 clean-room meets these criteria. There is no direct equivalent in AS 1386 to a class B environment, however, the counts accepted for an AS class 350 room are the same as for a class B room 'in-operation'. Acceptable alternatives are a Class 3.5 / Class A clean-room or an isolator.

For the testing of potentially dangerous drugs an appropriate laminar flow cabinet with a protective air curtain or an isolator should be used.

TABLE 6: CLEAN-ROOM ENVIRONMENT CLASSIFICATIONS

Environment	**European (PIC/S)**			**Australian (AS 1386)**	
	Class	number of particles ≥0.5 µm / m^3		**Class**	Number of particles ≥0.5 µm / litre
		at rest	**in operation**		**in operation**
Laminar flow cabinet; Isolator	A	3,500	3,500	3.5	3.5
Clean-rooms	B	3,500	350,000	not defined	
	C	350,000	3,500,000	350	350
	D	3,500,000	not defined	3,500	3,500

The testing work zone should provide sufficient space and material should be set out so as not to disrupt laminar air flow.

Air supply

Air supplied to the environment should be provided through terminal HEPA filters that should be fitted with audible and/or visual alarms to indicate pressure drop across the HEPA filters. There should be a pressure differential of 10 to 15 Pascals (guidance value) between each of the areas, ie, ambient/airlock and airlock/test room. A minimum of 20 air changes per hour is expected. Prior to operator entry to the test suite, pressure readings should be taken and recorded from externally mounted gauges labelled to indicate the area served, acceptable

specification and whether or not the reading is absolute or differential. An automated continuous monitoring system is an acceptable alternative to manual reading.

Certification

The test environment, which includes the laminar flow cabinet or isolator, should be certified at least annually by a competent person for compliance with the specified conditions.

Airlock

Entry to the clean-room should be via an airlock where operators change into clean-room garments. The airlock should be designed to facilitate movement of the operator from the unclean to the clean end of the room without compromising the aseptic gowning procedure. A step-over bench is a suitable division between these areas. The airlock should contain a full-length wall mirror, gowning instructions and hand washing/drying facilities.

Clean-room fittings and surfaces

The clean-room should have a minimum of ledges and obstructions to flow of clean air. In general, fittings such as power outlets and light fittings should be flush with walls/ceiling surfaces and sealed to prevent the entrainment of unclean air. Surfaces should be smooth and impervious to the cleaning agents used. The joints between walls/ceilings/floors should be coved to facilitate cleaning. The intercom or communication system should be designed to allow hands-free use. Chairs, trolleys and such items should be designed to facilitate cleaning and be suitable for clean-room use.

There should be no extraneous equipment within the clean-room environment.

Ultraviolet lights may be fitted only in pass-through hatches. If there is more than one parallel tube they should be shielded from each other. They should be checked at least annually or whenever new lamps are fitted.

Cleaning, sanitisation and disinfection

There should be written instructions for daily, weekly and periodic cleaning and decontamination of the test suite. If an isolator is used, the method of disinfection/sterilisation should be specified. Disinfectants should be free of microbiological contamination, which may be achieved by aseptic filtration or use of a product-compatible terminal sterilisation method. All disinfectants and detergents should be monitored by testing for contamination.

All cleaning, sanitising and disinfecting procedures should have been validated for minimum contact time and efficacy.
Surfaces and operators' gloved hands should be disinfected regularly during the test session.

Environmental monitoring

Environmental monitoring should consist of air sampling, settle plates, surface monitoring and operators' gloved hand plates. Surfaces can be monitored by contact (RODAC) plates, films or swabs. The laminar flow area should be monitored as well as the background room area.

Written procedures should specify exposure duration, frequency and location of all monitoring and include appropriate alert and action limits for microbial contamination. The media used should be specified and the recovery of micro-organisms from surfaces and on media should be validated. Suitable disinfectant/cleaning agent inactivators may need to be incorporated in media.

Records should be maintained of the numbers and types of organisms isolated and results presented in a format that facilitates early detection of trends. Routine identification of environmental micro-organisms to at least the genus level should assist in detecting trends. If the identity of organisms from the environment is to be used as the basis for invalidating a sterility test and performing a repeat sterility test, then a sensitive method of identification such as molecular typing techniques using RNA/DNA homology will be expected.

Testing Ancillary to the Sterility Test

The exposure and manipulation of cultures increases the likelihood of environmental contamination. Testing associated with the sterility test but requiring the use of live micro-organisms (eg validation, stasis testing) should be carried out in laboratory facilities that are completely separate from the clean-room. In addition, such aspects of testing for sterility that necessitate the handling of live cultures should not be carried out in or adjacent to production areas.

Sterility Testing Operators

Training

Sterility testing should only be performed by personnel who have been trained, qualified and certified to perform the various tasks and procedures related to sterility testing. The examination of test and control containers during and at the end of the incubation period should be included as part of the operator training program. Personnel should undergo periodic re-certification, particularly when problems are detected during the course of routine environmental and negative control monitoring, or when operators perform the test infrequently.

The operator's testing technique should be monitored during every test session by use of negative product controls. Techniques used should be reviewed periodically to ensure that departures from aseptic practices do not develop. Personnel training should be documented and records maintained.

Clean-room garments

The sterility test operator should wear sterile clean-room garments that consist of a one-piece coverall suit, a head cover, a beard cover if applicable, overshoes, gloves and mask. The use of sanitised garments may be acceptable if the process has been validated and their use is not used to justify the performance of repeat sterility tests. These garments should be changed for each work session, or at least once daily if validated by personnel monitoring.

Each operator should be trained and certified in aseptic gowning procedures as part of training for sterility testing, and training records maintained. Records of sterilisation of garments should be kept. This may be in the form of a certification from an external supplier of clean-room garments.

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