Sterility Testing – Overcoming Difficult Products

White Paper

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Sterility testing isolator (Image: RSSL)

Introduction

The sterility test, in its most basic form, is a qualitative assay that is designed to detect the absence of viable microbial cells in or on a product. The pharmacopeia culture-based method is based on the premise that a single microbial cell, present within the portion of the product transferred into the culture medium, will grow geometrically provided that the conditions are optimal.

Optimal conditions relate to different types of microorganisms and to the state of the microorganism (in relation to whether the microbial cell is 'stressed' or 'sub-lethally damaged'). Conditions also relate to the growth media, in terms of available nutrients, pH, temperature, atmosphere and incubation time1.

The compendial sterility testing of biopharmaceutical products is based upon the addition of aliquots or membranes with the concentrated samples to different types of media.

This paper is designed to support pharmacopeial monographs with an emphasis on products that are difficult to test or to validate, offering a practical approach to sterility testing. For many of these products there is little in the way of pharmacopeial guidance.

Such products include those which contain antibiotics or preservatives and for which a neutralisation step is required. Reference is also made to other difficult products like creams, ointments and medical devices.

In presenting general guidance for such products, this chapter discusses practical approaches that can be taken. Examples of potentially difficult products include[2]:

- Mercurial compounds
- Antibiotics
- Turbid samples
- Medical devices
- Oily samples
- Catgut
- Radiopharmaceuticals
- Cell lines

Care must be taken when developing methods so that it can be demonstrated that any microorganisms present in the product can be recovered (within the limitations of the cultural based method as set out in the harmonised pharmacopeia).

Although the test for sterility is carried out under aseptic conditions, the sterility test is at risk to adventitious contamination. Hence, the environment in which the test is conducted must be controlled and designed in such a way so that contamination risks are reduced (and here greater protection is provided through an isolator)3. The precautions taken to avoid contamination must be such that they do not affect any microorganisms which are to be revealed in the test. The working conditions in which the tests are performed must be monitored regularly by appropriate sampling of the working area and by carrying out appropriate controls.

Sterility Test Methods

There are two principle methods of sterility testing as defined in the pharmacopoeias[4,5]. These are tested by:

- Membrane filtration
- Direct inoculation

Of these, membrane filtration is the method of choice. This is because all the contents of a small volume product, or at least half the contents of a large volume product, are passed through a membrane filter. Therefore, a much larger sample size is tested when compared to the direct inoculation method (where the amount of product can vary from 1-2 ml, to half the container contents). Furthermore, the method is more adept at overcoming interfering factors, as any microorganisms present are far more likely to be separated from potentially inhibitory substances in the product through the act of filtration. Should they remain, they can be eliminated by rinsing the filter. It is also common for membrane filtration systems to be enclosed, such as with the Steritest™ system (introduced in 1975)[6], which minimises the risk of contamination by reducing transfer steps[7]. The membrane filtration method is arguably less prone to false positives due to the use of an enclosed system.

Membrane filtration is the appropriate method for all aqueous, alcoholic, oily and solvent products that can pass through a sterile filter with a porosity of 0.45µm. The standard filter is manufactured from cellulose esters or other similar plastics. The filter acts to separate the product from any microorganisms – when the product passes through the filter, any microorganisms present are trapped within the filter matrix. A rinse solution (such as phosphate buffered saline, saline or Ringer's Solution) is used to remove any product residues (due to the risk of antimicrobial activity posed from the residues). Different standard rinse solutions are suitable for different product matrices.

Type	Characteristics	Application
Fluid A (USP) / neutral solution of meat or casein peptone (EP)	• 0.1% Peptone: source of carbon and nitrogen • pH 7.1±0.2 • maintained osmotic equilibrium	• Suitable as a general rinse buffer • Works well with most samples • Excellent to dissolve or dilute samples • Excellent to reconstitute commercial microorganisms • Excellent transport medium for microorganisms
Fluid D (USP)	• Fluid A + 1 ml polysorbate 80 (0,1%) Polysorbate 80: will neutralise some preservatives • Peptone: source of carbon & nitrogen • pH 7.1 ±0.2 • maintained osmotic equilibrium	• Suitable for testing specimens that contain lecithin or oil • Excellent for rinsing sterile pathways of devices • Works well with most antibiotics • Needed for rinse method testing of medical devices
Fluid K (USP) (neutral solution with emulsifying agent (EP))	• Beef extract and peptone: provide nutrients for recovery of injured and fastidious microorganisms • Polysorbate 80 at a concentration of 10 g/l (1 %) • pH 6.9 ±0.2 maintained osmotic equilibrium	• Suitable for testing specimens that contain petrolatum • Suitable for oils and oily solutions • Excellent for rinsing pathways of Medical Devices • Good for "difficult" sample to filter or to dissolve samples

Table 1: Different pharmacopeia recommended rinse solutions

This washing process is normally performed three times. The maximum number of rinses permitted in the pharmacopeia is 5 x 100 ml. Where this number of maximum rinses does not eliminate anti-microbial substances, a method variation is required (as set out later in this white paper).

When testing solid products, these materials require dissolving in a suitable solvent. Such solvents include the solvent provided with the preparation, water for injections or saline – the method suitability test will verify the suitability of the solvent. Different techniques are required for the testing of oily substances, creams and aerosol canisters.

On completion of the product filtration and filter rinsing, the filter is divided into two portions (or more than one filter is used, as in the widely used Steritest polycarbonate filtration system).

Culture media is added to these separate filter membranes. This is so that any microorganisms trapped in the filter membrane (following incubation at a suitable temperature) will replicate. Two culture media are used. The pharmacopoeias recommend fluid thioglycolate medium, incubated at 30-35°C to isolate bacteria (aerobic and anaerobic), and soy-bean casein digest medium (commercially known as tryptone soya broth), incubated at 20-25°C to isolate aerobic bacteria and fungi. The volume of media added must be the same as the volume used for the method suitability test.

Inspecting a bottle from the direct inoculation test (Image: Tim Sandle)

Many products will not readily filter (such as protein-based products which will block the filter pores), or are so inherently anti-microbial that the membrane filtration method is inappropriate. In these circumstances, the direct inoculation method is used.

Direct inoculation may also be preferred over membrane filtration if the membrane filtration method simply cannot be validated. When the direct inoculation method is selected, the laboratory should be able to justify why it has selected this method over the membrane filtration technique. The direct inoculation technique involves the addition of a portion of the product to two different culture media (the same media as per the membrane filtration technique).

An isolator undergoing construction (Image: Tim Sandle)

The amount of product transferred into the media was, in previous versions of the pharmacopeia, half the contents of the product vial to each culture medium (for product between 50 mg and 300 mg) and the entire contents for products less than 50 mg. With the harmonised text, this changed to the volume being not more than 10% of the volume of the culture medium (given that a typical bottle of prepared culture media is 100 ml, this aliquot is 10 ml per bottle).

For the direct inoculation technique, products which have anti-microbial activity must be neutralised before a portion of the product is added to the culture medium. This is performed either by the addition of a neutraliser or by the dilution of the product.

In addition to the established culture-based methods, there are different rapid methods available, including[8]:

- Respirometry pressure sensing technologies. Detection of metabolic activity is determined by pressure transients relating to gaseous exchanges within the closed culture vessel as a result of microbial respiration
- Growth-based carbon dioxide detection, where detection indicates the presence of viable microorganisms
- ATP bioluminescence, where luciferin / luciferase enzyme reagent catalyses the conversion of microbial adenosine triphosphate (ATP) into adenosine diphosphate (ADP) and light
- Viability staining and solid phase cytometry. Post-staining, detection of microbes is achieved using digital fluorescent microscopy at specific excitation and emission wavelengths

Preparing a sterility test in an isolator (Image: Tim Sandle)

While not directly discussed in this white paper, challenging products may be easier to test using one of the emerging technologies. However, regulatory approval, supported by considerable validation data, is required in order to change from the pharmacopoeia method. In terms of the future, the use of automated

sterility testing systems should enable more rapid and more reliable detection of microbial contamination by using specific indicators and consequent automated analysis for the detection of microbial growth[9].

Method Validation (Method Suitability)

Unlike some analytical assays, the culture based sterility test is not 'validated' as a method in itself, rather the validation element is a test of the culture media in the presence of the product. This is in order to show that, should microorganisms be present, the product does not cause inhibition of the microbes. It is for this reason that the pharmacopeia does not use the term 'test validation' and instead uses the phrase 'method suitability test'. This testing is also commonly referred to as the bacteriostasis / fungistasis (B/F) test[10].

Key criteria to consider before embarking on the method suitability exercise are:

- Filterability
- Chemical compatibility
- Rinsing fluids and volumes
- Potential inhibition issues. To be addressed by:
  - Dilution
  - Chemical neutralisation
  - Filtration and rinsing
  - Enzyme activity
- Membrane compatibility
- Quantity of samples to be tested

It is important to determine if the article to be tested for sterility contains elements that will interfere with the growth of microorganisms within the growth media used for the assay. This needs to be conducted for each product or groups of products.

Validation involves the use of different microorganisms. These are described in the pharmacopeia and they are the same as those used for the media growth promotion testing, namely as per Table 2:

Species name	Type	American Type Culture Collection (ATCC) reference
Staphylococcus aureus	Aerobic bacterium	ATCC 6538
Bacillus subtilis	Aerobic bacterium	ATCC 6633
Pseudomonas aeruginosa	Aerobic bacterium	ATCC 9027
Clostridium sporogenes	Anaerobic bacterium	ATCC 19404
Candida albicans	Fungi	ATCC 10231
Aspergillus brasiliensis	Fungi	ATCC 16404

Table 2: Test panel for the sterility test method suitability test

Alternative culture collections may be used to those listed in Table 2, provided that equivalence with the ATCC strains can be shown. Readers unfamiliar with the last species of fungus listed should note that until 2008, *Aspergillus brasiliensis* was known as *Aspergillus niger*.

From the above list, it can be seen that the different types of microorganisms are included (A Gram-positive coccus, a Gram-positive rod, Gram-negative rod and fungus). These microorganisms represent each of the applicable morphological groups that might be found in the pharmaceutical manufacturing environment. In addition, as with other 'validations' of pharmaceutical microbiology methods, there is a tendency to supplement the recommended pharmacopeial cultures with isolates from the manufacturing environment for the validation test panel. Whether this is entirely necessary and offers anything more than the pharmacopeial strains is questionable. However, there is an expectation amongst many regulators that representative 'wildtypes' or environmental isolates are included.

In addition, the FDA guidance document on Sterile Drug Products recommends that if a sterility test failure has previously occurred, the isolate responsible for the contamination is additionally used to challenge the culture media for release purposes going forwards.

Each of the cultures used must be no more than five passages removed from the original supplied culture from the culture collection (or seed lot). This is presumably to avoid any phenotypic or genotypic (genetic drift) changes which might arise from successive subcultures, as well as associated risks of contamination and loss of cell viability[11].

Validation Method

The suitability method is similar to the growth promotion test conducted on the test media, with modifications made to reflect the test method. The sterility test validation involves, for each type of microorganism listed further down this page, the following:

- Membrane filtration method: after transferring the contents of each final product vial / bottle through the membrane, less than 100 CFU is added to the final portion of the diluent used to rinse the filter
- Direct inoculation method: after transferring the contents of each final product vial / bottle into the culture medium, less than 100 CFU is added to the test culture media bottle

Fungal plates (Image: Tim Sandle)

It is important, when conducting the test, to note the volume of culture media used. The volume of media added sets the volume to be used for the routine sterility test.

The required microorganism: media combinations as shown below:

Microorganism	Media
Staphylococcus aureus subsp. aureus (ATCC 6538)	FTM, SCDM
Bacillus subtilis subsp. spizizenii (ATCC 6633)	FTM, SCDM
Pseudomonas aeruginosa (ATCC 9027)	FTM, SCDM
Clostridium sporogenes (ATCC 19404)	FTM
Candida albicans (ATCC 10231)	SCDM
Aspergillus brasilensis (ATCC 16404)	SCDM

Where:

- FTM = fluid thioglycolate medium
- SCM = soyabean casein digest medium (commonly called tryptone soya broth)

These organisms are commonly supplemented with environmental isolates. With environmental isolates, decisions are required relating to:

- The number of isolates to include with each method suitability test
- The reason for selection based on reviews of facility microbiota. Here, it may not be necessary to include organisms that are very similar to the recommended organisms
- The recovery time. For example, is the same five-day recovery time required for test organisms adopted?
- The frequency of rotation, in terms of changing environmental isolates. Furthermore, does a need to change trigger the need for test revalidation?

At the same time as challenging the media in the presence of the product, positive controls must be prepared. One positive control must be a total viable count, such as a pour plate, to verify that the challenge inoculum was acceptable. The second control is an article of media for each microorganism which has not had contact with the product (in essence, a re-run of the growth promotion test).

One difference from the standard growth promotion test is that all challenged articles are incubated for up to five days, irrespective of whether the challenge is from a bacterium or from a fungus.

This is a far shorter time than the 14 days required by the pharmacopeia. This is because the challenge cultures are considered to be 'healthy' (based on their cultivation of highly nutritious media within the laboratory) compared with the aim of the sterility test itself, which is to recover organisms living in a low nutrient environment and hence undergoing cellular stress leading to repressed growth.

Alternatively, the organisms may be sub-lethally damaged and require a longer recovery time, while other organisms may be in a 'resting state' such as those that can form spores.

At the end of the incubation period, the challenged articles are to be inspected for turbidity. If copious growth of microorganisms is obtained after the incubation, visually comparable to that in the positive control (media without product), it can be concluded that either the product possesses no antimicrobial activity under the conditions of the test, or such activity has been satisfactorily eliminated.

However, if clearly visible growth is not obtained in the presence of the product to be tested, then it can only be concluded that the product possesses antimicrobial activity that has not been satisfactorily eliminated under the conditions of the test. From this position a modification to the test is required.

Importance of visual inspection (Image: Tim Sandle)

Often the use of multiple rinses (in the case of the membrane filtration test), the use of different types of filters (such as cellulose acetate or cellulose nitrate) or the addition of neutralisers to media is required in order for products to pass the test.

Re-validation

The pharmacopeia does not mention the need to conduct re-validation of the products used with the sterility test for situations where the product has remained unchanged in terms of its formulation or with the process used to manufacture it. Certainly, a major change to a product formulation should trigger consideration of re-validation through a change control process, as would, what the pharmacopeia describes as, "whenever there is a change in the experimental conditions of the test". However, where no changes have taken place with either the formulation of the product or with the test methodology, the need for re-validation is debatable. Some organisations do, however, elect to re-validate the products on a periodic basis.

Hence, we can present some reasons to re-validate as:

- Change to a product formulation
- Change of test method (direct inoculation to membrane filtration)
- Change to membrane filters
- Change to media formulation
- Change of sterility test kit manufacturer
- Where there is more doubt, is where there is a:
- Change to the test environment (e.g. cleanroom to isolator)
- Technology transfer (such as from a laboratory in a pharmaceutical company to a contract test facility)
- Change with facility microbiota, in terms of different environmental organisms being recovered from the aseptic processing environment

With the latter section, each facility will need to reach a position appropriate to their needs.

Cellular Products

The sterility test method described previously does not directly apply to cellular products and an alternative sterility test method is recommended (in the European Pharmacopeia, monograph 2.6.27). For this method, two culture media are used, and the cells are inoculated into it using either manual or automated methods. The media are incubated 35-37°C for no less than 7 days.

The challenge microorganisms for the media growth promotion tests differ slightly to the standard sterility test. These are presented in Table 3.

Aerobic medium	American Type Culture Collection (ATCC) reference
Staphylococcus aureus	ATCC 6538
Bacillus subtilis	ATCC 6633
Pseudomonas aeruginosa	ATCC 9027
Candida albicans	ATCC 10231
Aspergillus brasiliensis	ATCC 16404
Anaerobic medium	**American Type Culture Collection (ATCC) reference**
Clostridium sporogenes	ATCC 19404
Bacteroides fragilis	ATCC 25285

Table 3: Microorganisms required for the verification of cell culture sterility tests

Alternative culture collections may be used, provided that equivalence with the ATCC strains can be shown.

With method suitability to determine the limit of detection, the test is carried out using the preparation deliberately contaminated to different degrees with the following microorganisms, chosen for the likelihood of contamination and their growth requirements:

- Aspergillus brasiliensis, ATCC 16404
- Bacillus subtilis, ATCC 6633
- Candida albicans, ATCC 10231
- Clostridium sporogenes, ATCC 1940
- Propionibacterium acnes, ATCC 11827
- Pseudomonas aeruginosa, ATCC 9027
- Staphylococcus aureus, ATCC 6538
- Streptococcus pyogenes, ATCC 19615
- Yersinia enterocolitica, ATCC 9610

An additional concern with cellular products is with mycoplasma contamination. Rapid microbiological

methods suitable for examining cellular products include growth based carbon dioxide sensor systems. With such technologies, during microbial growth, carbon dioxide in a closed container accumulates. The test is designed to allow the gas to diffuse into a colorimetric sensor. Here, hydrogen ions interact with the sensor resulting in a decrease in pH, causing the sensor to change to a different colour. The generation of carbon dioxide is used to demonstrate the presence of growing microorganisms.

Combination Products

The sterility test of combination products is not straightforward. The main problem is whether a product is a 'medical device' or a 'pharmaceutical'. Combination products are therapeutic and diagnostic products that combine drugs, device and / or biological products, as defined in 21 CFR 3.2 (e)[12]:

- Single-entity: a product comprised of two or more regulated components that are physically, chemically or otherwise combined or mixed as a single entity
- Kits: two or more separate products packaged together (e.g. drug and device products)
- Cross-labelled: provided separately but intended for use together where both are required to achieve the intended use and where cross-labelling is needed

Examples include absorbable collagen sponges, catheters and pre-filled syringes.

If the product is considered to be a medical device, then the test method outlined in ISO 11737-2: 2019 should be followed[13].

This standard allows for either a direct inoculation method or a rinsing method followed by membrane filtration to be used. In relation to both possible methods, only one test medium is required (soy-bean casein digest medium incubated at 28-32°C for 14 days).

Unlike the sterility test method described in the pharmacopoeia, the ISO standard does not mention the need for method suitability testing. For products classified as pharmaceuticals, then the pharmacopeia sterility test method is followed.

The important choice to be made rests on deciding how many items to test (or with large items, how many portions to test), the most suitable elution technique and whether a surface active agent is needed in combination with mixing and shaking to recover any microorganisms that might be present.

Practical Approaches for 'Difficult' Products

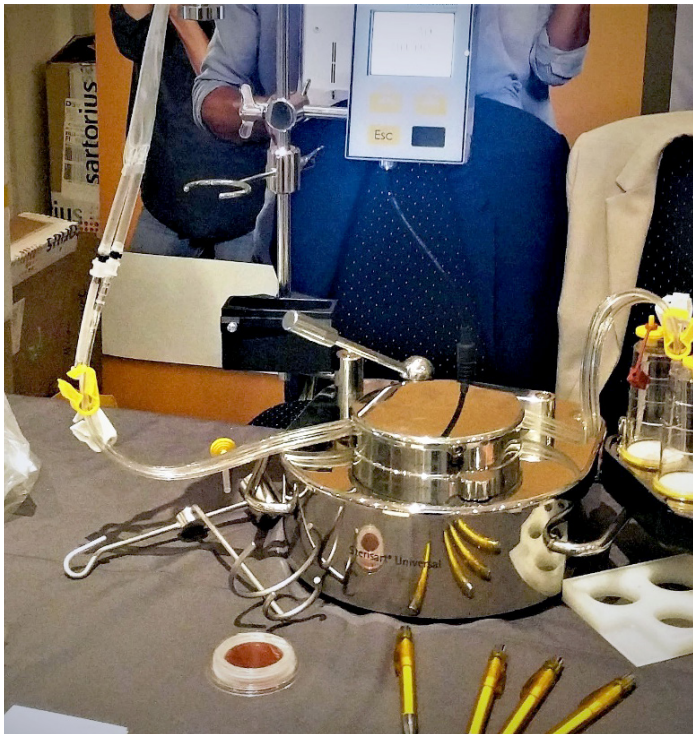
Some products (or the preservative that is added to them) possess an antimicrobial activity. These products will not pass the sterility test validation without some form of modification or manipulation[14]. It may be that only some microorganisms will be inhibited and not others (for example, in this author's experience, Aspergillus brasilinensis is the most resistant of the standard set of validation microorganisms to antimicrobial substances and a hostile cultural environment). Such a situation where only some microorganisms are recoverable is unacceptable and a method needs to be established whereby each of the required test panel of microorganisms exhibits growth.

This part of the paper outlines some general variations to technique that can be adopted for dealing with products that will not pass the sterility test validation[15]. The list is not necessarily in order of priority and the laboratory will need to consider the cost and time implications of each. It may also be that some manipulations are easier to perform in a conventional cleanroom than they are inside an isolator, which may also have a bearing on the method selection. Importantly the validation should be carefully designed to consider the type of product to be examined.

Membrane Filtration

a) Type of membrane filter

There are different types of membrane filter that can be used for the sterility test. These are divided by filter properties – hydrophobic or hydrophilic – and by the primary material of manufacture: nylon, cellulose acetate, cellulose nitrate or polycarbonate.



Membrane filtration apparatus (Image: Tim Sandle)

All standard filters used in the sterility test have a porosity of 0.45µm with low product binding characteristics. For a 'standard' aqueous based product, hydrophilic filters are the most commonly used (such as those manufactured from mixed esters of cellulose). Hydrophilic filters can be wetted with virtually any liquid, allowing the liquid to pass through the filter effectively.

Hydrophobic edged filters are widely used for the membrane filtration of antibiotics (such as those manufactured from mixed esters of cellulose, polyvinylidene difluoride or polycarbonate). This is

because the use of a conventional hydrophilic filter to test antibiotics can lead to the antibiotic remaining at the periphery of the membrane (which would affect bacterial growth as the material is difficult to remove through standard rinsing)[16].

If antimicrobial residues remain then this can lead to a false, negative result. Ideally this will be picked up at the validation stage, as the product should not pass the validation test due to inhibition of the challenge microorganisms. A hydrophobic filter overcomes this phenomenon by minimising the antimicrobial residues at the filter edge (which are difficult to rinse out).

A hydrophobic filter can additionally help to separate out microbial cells from the product. The rinse solution then, ideally, rinses away product residues, leaving only microbial cells trapped in the filter matrix.

The two main filter material types are cellulose nitrate and cellulose acetate. Sometimes the key to a successful validation is as straightforward as selecting the correct filter type. Cellulose nitrate filters are used for testing aqueous, oily and weakly alcoholic solutions, whereas cellulose acetate filters are preferred for the testing of strongly alcoholic solutions.

b) Pump speed

When undertaking the membrane filtration test, controlling the pump speed can assist with the filtration of certain products, such as reducing the amount of foaming or reducing the tendency for the filter to block. It is unlikely that variations to the pump speed alone will make a significant difference to whether a material can be successfully tested or not. It is important that the pump speed is recorded when undertaking test validation, as this can avoid problems when the method is transferred for routine testing.

c) Type and number of rinse solutions

If inhibition cannot be overcome by the selection of the membrane filter alone, then the rinsing of the filter can often overcome antimicrobial effects, especially where product becomes bound to the membrane filter. The common rinse solutions used in the sterility test are saline, peptone water, phosphate buffered saline, or Ringer's Solution. Each of these solutions is used for basic rinsing when no neutralisation is required. These solutions provide an osmotically balanced environment, which can aid microbial recovery.

i) Saline is a general term for a solution of sodium chloride (NaCl). For the sterility test, phosphate buffered saline is more commonly used than standard saline.

ii) Peptone water contains peptone as a source of carbon, nitrogen, vitamins and minerals. Peptones are derived from animal milk or meat digested by proteolytic digestion. In addition to containing small peptides, peptone material includes fats, metals, salts, vitamins and many other biological compounds. For peptone water, sodium chloride is added to maintain the osmotic balance. A typical formulation is[17]:

- Peptone: 10g
- Sodium Chloride 5g
- 1 litre of water
- Final pH: 7.2 ± 0.2 at 25°C

iii) Phosphate buffered saline is a water-based salt solution containing sodium chloride, sodium phosphate and, in some formulations, potassium chloride and potassium phosphate. The buffer's phosphate groups help to maintain a constant pH.

iv) Ringer's Solution is the name given to a solution of several salts dissolved in water for the purpose of creating an isotonic solution relative to the bodily fluids of an animal. Ringer's Solution contains sodium chloride, potassium chloride, calcium chloride and sodium bicarbonate, with the latter used to balance the pH.

Microbiological culture media (Image: Tim Sandle)

Where neutralisation is required, Proud and Sutton found that a 'Universal Diluting Fluid' (UDF), based on Dey-Engley neutralising broth, was the optimal solution for neutralising antimicrobial activity[18]. Dey-Engley medium was formulated to inhibit the activity of a wide range of disinfectants while allowing bacterial growth[19]. The medium has since undergone different modifications to enhance its effectiveness. Studies have shown UDF to be effective against such compounds as thiomersal, benzalkonium chloride, biguandies and so on.

Variations can be made to the rinse solution through the addition of other neutralisers. Common general additives include polysorbate-80 or the surfactant Triton X-100. Polysorbate 80 is a non-ionic surfactant and emulsifier derived from polyethoxylated sorbitan and oleic acid[20]. Triton X-100 ($C_{14}H_{22}O(C_2H_4O)_n$) is a nonionic surfactant which has a hydrophilic polyethylene oxide chain and an aromatic hydrocarbon lipophilic or hydrophobic group[21]. There is some uncertainty as to whether this substance is toxic to some microorganisms and would itself create a false negative.

Microbiological culture media (Image: Tim Sandle)

For antibiotics, the main neutraliser is penicillinase. Penicillinase is a specific type of β-lactamase, showing specificity for penicillins. Beta-lactamases are enzymes produced by some bacteria and are responsible for their resistance to beta-lactam antibiotics like penicillins, cephamycins and carbapenems (ertapenem)[22]. When added to a rinse fluid, a beta lactamase cleaves the beta-lactam ring (thus destroying the activity of the antibiotic).

In addition to the above-mentioned neutralisers, USP <1227> provides a listing of some of the more popularly used neutralisers.

Antimicrobial effects can be overcome further by varying the number of rinse solutions. The pharmacopeia places a limit on the number of rinse solutions that can be performed (this is 5 x 500 ml after a recommended 3 x 100 ml has been attempted). The pharmacopeia did once allow dispensation for a product to be released without validation if this is acceptable with the Regulatory Authority, however the text in the harmonised pharmacopeia states: "Modify the conditions in order to eliminate the antimicrobial activity and repeat the method suitability test." In other words, continue to modify the method until the product can be successfully validated.

Even by varying the amount of rinse solutions or the formulation of the rinse solution, some products remain difficult to remove through rinsing due to their tenacious attachment to the filter membrane. An example of one particularly tenacious product is erythromycin lactiobionate.

Direct Inoculation

a) Type of neutralising agent

For the direct inoculation test, there are different neutralising agents that can be added to the culture medium (or even, with some degree of caution, directly into the product) and can therefore be used to inactivate different antimicrobial compounds. Some products require specific neutralisation agents, for others, multipurpose agents like lecithin or polysorbate-80 ('Tween') can be used.

Table 4 shows some examples of neutralising agents appropriate for different antimicrobial agents (these are applicable to both the direct inoculation and the membrane filtration test)[23]:

Antimicrobial agent / product	Neutralising agent
Benzalkonium chloride 0.01%	0.5% Lecithin and 3% polysorbate-80
Chlorohexine	Lecithin and polysorbate-80
Parabenz	5% Polysorbate-80 or 0.07% lecithin and 0.5% polysorbate-80
Mercurial compounds	Thioglycollate / sodium thiosulphate / thioglycollate with cysteine
Azide	Azolectin
Sorbic acid	Dilution and polysorbate-80
Collageb implant	3% Polysorbate-80
Organic acids	Polysorbate-80
Penicillin / cephalosporins	Penicillinase (β-lactamase – volume determined from antibiotic assay). Considered less effective for cephalosporins – membrane filtration recommended
Chloraphenicol	Chloramphenicol acetyltransferase
Sulphonamide	P-aminobenzoic acid

Table 4: Neutralisation agents

b) Dilution

The dilution of some products prior to direct inoculation can overcome antimicrobial properties, as can varying the volume of the culture media used. For culture media volumes, the USP (until version 27) allowed up to 2000 ml of culture media to be used if other attempts at neutralisation are not successful (this remains important in order to allow sufficient air space in the tryptone soya broth when selecting the culture media volume).

If 2000 ml was not successful, the USP then allowed the sterility test to proceed using a media volume of 2000 ml (provided the laboratory can be granted acceptance by the Regulator). It may be possible to argue for a volume greater than 2000 ml if this is required for the immersion of a medical device.

In contrast, the Ph. Eur. and the 2004 version of the USP (#27) onwards, contains no set dilution limit provided that the "volume of the product is not more than 10% of the volume of the medium." The problem with increasing volumes is that there is a danger of this resulting in a low recovery of any low-level contamination or the possibility of increasing the likelihood of there being a false positive through increased manipulations.

For medical devices, large volumes of media may be required.

Alternatively, instead of increasing the volume of the culture media, an inhibitory effect can be overcome through the dilution of the product.

An example of varying the product dilution is the dilution of benzyl alcohol or phenol, which requires a 1:50 dilution with sterile water. Furthermore, the type of diluent used can have an impact upon the extent of the antimicrobial activity. The use of some solvents or solutions with neutralisers can be more effective than simple dilution or reconstitution with sterile water.

Turbid vials (Image: Tim Sandle)

c) Turbid samples

Turbid samples present a problem, especially when the direct inoculation method is used. For normal sterility testing, turbid samples require subculturing. When this occurs is a matter of debate. The pre-2012 version of the FDA CFR 610 described this as taking place 3–7 days after the initial test and then re-incubating the subcultured product for an additional 7 days alongside the original sterility test (therefore the total test time became 14 + 7 days).

In comparison, the Ph. Eur. and the USP (from edition #27 onwards), described this taking place after the end of the standard sterility test incubation (i.e. after 14 days have elapsed).

With no direct reference in the current CFR to specific test requirements for the cultural method, the subculture step is normally undertaken after the final read as according to the pharmacopoeia.

The incubation time for the 'second' test (the turbid sample subculture) is not clearly specified. The harmonised pharmacopeial text reads: "transfer portions (each not less than 1 ml) of the medium to fresh vessels of the same medium and then incubate the original and transfer vessels for not less than 4 days." The laboratory will need to determine an appropriate incubation time. To validate this, either a similar subculture is required to demonstrate whether there is microbial growth at the end of incubation into broth or plating out (because microbial growth may not be seen in a broth that is rendered turbid by the product itself).

For both techniques

a) Pre-treatment of the product

Some products, particularly solids or articles, require manipulation prior to filtration or direct inoculation. Typically, this involves either dissolving the product in water (if the product is water soluble or has been freeze-dried) or dissolving with a solvent (such as with products like creams or water insoluble substances). For direct inoculation, either dissolving or adding the solid (or disassembling the article) directly into the culture media is a further pre-treatment that can be considered. The addition of a heating step can either facilitate or speed up the dissolution. Variation to these approaches can influence the success or otherwise of the validation. However, such approaches can often be variable and it is important to consider all possible differences in product volumes in the validation exercise.

b) Use of non-standard culture media

Some products are not testable using the culture media described in the pharmacopoeias, hence alternative or modified media may be used. With alternative media, some users select Fluid Sabouraud Media instead of, or along with, tryptone soya broth if the potential for there being fungal contamination is high. For the testing of water assumed sterile, some data indicates that the use of R2A is preferable to tryptone soya broth. This is due to the medium's ability to yield higher numbers of

bacteria from water (the bacteria being held in low nutrient environments and in a stressed or damaged state and therefore may not be able to multiply in the nutrient rich tryptone soya medium). To go down this path would be a variation from the pharmacopeia and this would need justifying and validating.

An example of modified media is with the testing of penicillins where the addition of penicillinase to media is required (β-lactamases as discussed). Furthermore, the testing of medical devices is often performed using alternative thioglycollate medium (this approach is described in the USP, but it is not listed in the Ph. Eur. because the European Pharmacopoeia does not extend to cover medical devices). The alternative medium can be one without agar and resazurin sodium solution.

A further modification is with the addition of neutralisers to culture media in order to overcome antimicrobial properties. Here the growth promotion properties of the modified media must be demonstrated prior to undertaking the main sterility test validation.

The different validation approaches have been summarised in the below flow chart (Figure 1).

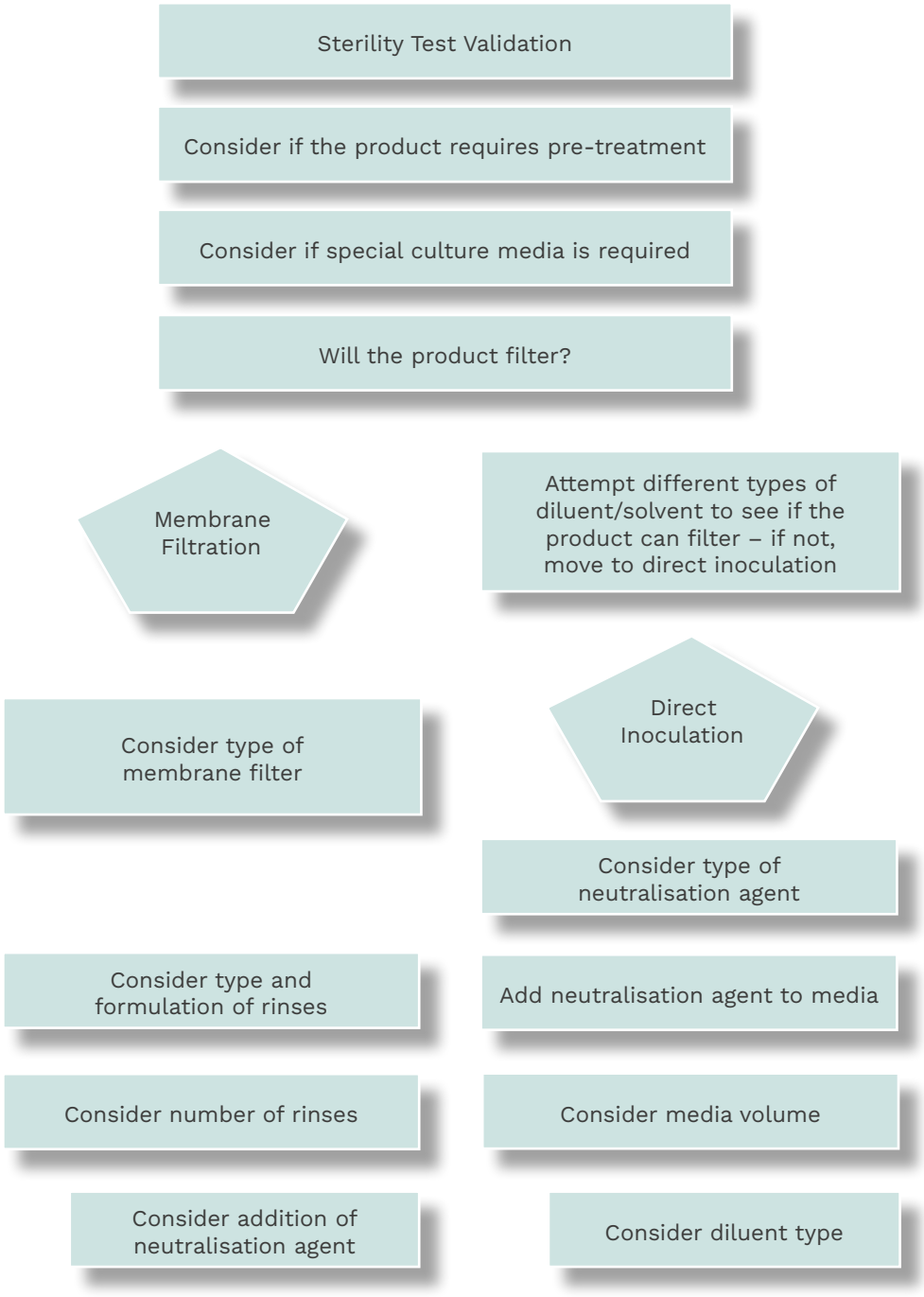


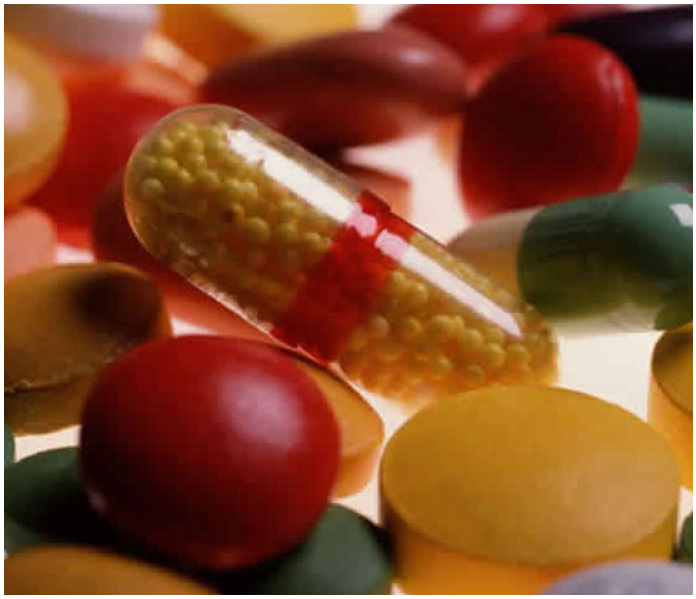
Figure 1: Sterility test validation decision tree

Some Examples of Testing 'Special' Products

The previous section has examined general variations to the sterility test validation method. This section of the white paper looks at some specific 'difficult' product examples[24].

Antibiotics

When validating antibiotics using the membrane filtration technique, it is especially important to pre-wet the filter with a rinse solution (such as saline) and not to let the filter dry-out during testing. The test volume must be kept to an acceptable minimum. For small volume products it is permissible to pool an appropriate number of samples in a single bottle and filter the contents. As discussed, it is important to use a hydrophobic edged filter to ensure that no product residues remain as such residues can potentially cause inhibition of microorganisms[25]. The other important consideration is with the use of neutralisers[26], as outlined previously.



Antibiotics (Image: Creative Common Library)

Options for testing antibiotics include the following and may be used in combination[27]:

- Adding an antibiotic neutralising / inactivating agent to the broth media
- Increasing the concentration of the antibiotic neutraliser / inactivator in the broth media
- Physical separation of the organisms from the antibiotic (i.e. membrane filtration). Here an alternate membrane filter media could be considered (for example, polyvinylidene difluoride [PVDF] or polyethersulfone [PES]). This is because cellulose-based membranes tend to bind antibiotics. If the microorganism(s) do not grow on the antibiotic-filtered membrane, there is still activity within the membrane. This may be eliminated by changing the filter material (e.g. PVDF or PES)
- Adding chemical neutralisers to the rinse medium
- Increasing the volume of the rinse
- Warming the product

Oily samples

An oily product, such as an eye ointment, can prove difficult to test because any microbial cells present can become embedded in the matrix of the product. In order to remove any microorganisms that may be present, the use of a solvent or an emulsifying agent is required (for example, isopropyl myrisate, polysorbate-80 or light paraffin). When conducting the test, the oil should be allowed to penetrate the membrane at its own weight and then be filtered by applying the pressure or suction slowly.

Some general guidance is:

- Oils of a low viscosity: run through a dry membrane
- Viscous oils: dilute with a diluent, filter slowly and then rinse. Sometimes a membrane filter with a larger surface area than the standard 47mm can aid filtration (this is not possible with most commercial sterility test kits)
- Fatty oils: dilute to 1% in isopropyl myristate at a temperature of 40-44°C, filter as rapidly as possible and wash the membrane speedily. Isopropyl myristate is the ester of isopropanol and myristic acid
- If the product gels in different emulsifying agents, vortexing can be attempted. If this does not work, the dilution can be increased. If this does not work, different emulsifying fluids should be used, for example saline with 0.5% polysorbate 80[28]

Once the test is in incubation, cultures essentially comprising of 'oily preparations' should be shaken gently every day. With the fluid thioglycolate, medium shaking or mixing must be restricted to a minimum level to maintain anaerobic conditions.

Other ointments and creams

Generally, ointments and creams require dilution at approximately 1 in 10 by emulsifying with a suitable emulsifying agent in a suitable diluent (such as polysorbate 80 or liquid paraffin). This provides an aqueous vehicle capable of dispersing the test material homogeneously throughout the 'fluid mixture'. Furthermore, this will improve contact between the sample and the culture medium. If this is unsuccessful, emulsifying agents can be added to the culture media. Examples include adding 10g/L of polysorbate-80 or 1g/L of (p-tert-octylphenoxy) polyoxyethanol.

Following the addition of the emulsifying agent, 10 ml of the fluid mixture should be mixed with 80 ml of the medium and subsequently tested using the standard method.

Anti-cancer treatments and radiopharmaceuticals

Anti-cancer drugs are exceedingly difficult to test using conventional membrane filter techniques because the drugs are readily adsorbed into the membrane and cannot be easily removed. Consequently, many of these types of drug products are tested by direct inoculation.

The sterility testing of radiopharmaceuticals poses some problems. Firstly, the total amount of material available for testing is usually limited and may be highly radioactive. Secondly, the shelf life of the material is also limited. Thus, for some radiopharmaceuticals it may not be possible to obtain the results of the sterility test before the product is released. However, cGMP requires that the test be conducted as a monitor for the manufacturing process, or alternatively, that a system of controls is in place and a justification produced not to undertake the sterility test[29].

Where the substance is highly radioactive and the sterility test is needed, a modification to the sterility test is often required. For example, a radioactive indicator method for the detection of viable bacteria based on the monitoring of 14C02 released from growth media containing 14C-labeled substrates, is a possible alternative[30].

Implants

Solid substances are difficult to test for sterility. In the case of implants the therapeutic protein / peptide needs to be reverted into a fluid monomer so that it can be adapted for testing and so that any trapped microorganisms in the matrix and can eluted.

Sterile Aerosols

The testing of aerosols in cans can prove problematic. Until commercially adaptable membrane filter units became available, the main means of testing was to freeze the containers in an alcohol-dry ice mixture for one hour. The container was then aseptically opened (by puncturing) and the contents transferred to a sterile pooling vessel (by expelling the contents) for testing. The main concerns were to avoid alcohol from entering the vessel, to avoid adventitious contamination and the danger of the can exploding.

Although the above method is still used, the advent of commercial membrane filtration units allows the connection of the nozzle directly to the aerosol canister and the transfer of the contents directly to the filtration unit. This provides a greater assurance of asepsis.

Cell lines

A different approach to the testing of cell lines is required compared with other 'products'. The difference is primarily in the selection of different culture media and different incubation conditions, depending upon the type of cells that are being tested. The use of an alternative to the thioglycollate medium is advisable in some cases due to reports that conventional FTB is toxic to some damaged cells[31]. A possible approach for sterility testing of cell lines is as follows:

a. Thaw the different cell lines to be tested and pool
b. Centrifuge in order to separate the culture from the cells
c. Inoculate 2–3 drops (0.5 ml) of the culture into a variety of different media. The type of media used will depend upon the application and cell culture type (see Table 5 for examples).

Culture Media	Temperature	Incubation: (Min. #Days)
One Blood Agar plate (aerobic)	37°C	14
One Blood Agar plate (anaerobic)	37°C	14
Two Thioglycollate Broth tubes	37°C and 23°C	14
Two Tryptone Soya Broth tubes	37°C and 23°C	14
Two Sabouraud Broth tubes	37°C and 23°C	21

Table 5: Culture media for sterility testing cell lines

The difficulties posed by mammalian cell cultures tend to increase with the density of the cells. This can be addressed by using sterile lysis solutions containing detergents. Lysis protocols have been developed by manufacturers to enable membrane filtration to take place in some instances.

The above approach will provide a level of assurance that there is no gross contamination from bacteria or fungi. Given the nature of the cell cultures, the inoculated media should be inspected after 48 hours incubation (growth is likely to have occurred within this timeframe).

A risk to cell cultures arises from mycoplasmas. Testing for mycoplasmas requires specialist culture media and a nitrogen rich atmosphere (where fungal contamination poses a significant risk). Post-test examination requires the use of a fluorescent microscope. Alternatively, biochemical tests are also available.

Fibrin Sealant

The reconstitution of freeze-dried products can be highly variable, and the amount of diluent added, as well as the types of diluent are of importance. Other variables include the degree of agitation (or stirring speed) and the maintenance of the correct temperature. This author has noticed that even +/-1°C in the reconstitution of a protein based product (in this case, a fibrin sealant) can result in coagulation and thus to an invalid test.

Dressings

For dressings, the entire product does not need to be tested. The recommended amount is normally 100–500mg. The portion selected should be the part of the dressing considered to be the most inaccessible to the sterilant. It is also permissible to pool dressing.

Solid articles that cannot be tested using the standard methods

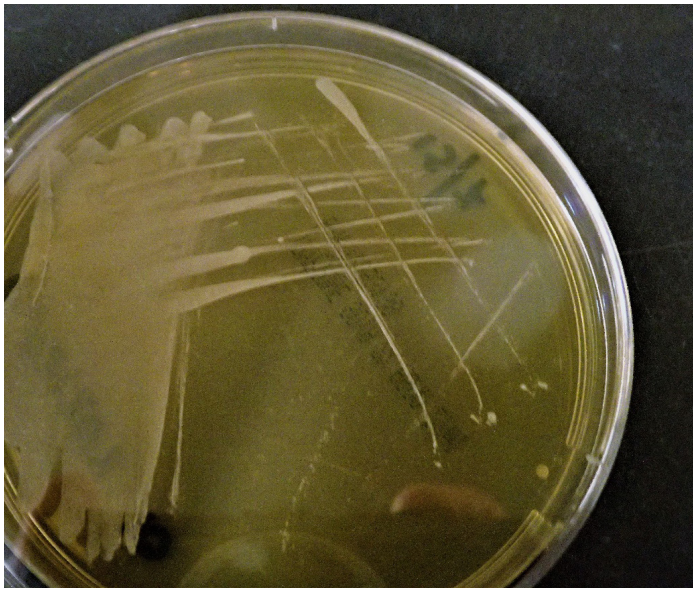
Solid articles like medical devices are normally examined by direct inoculation (immersion into broth media). For the testing of such devices, where the articles are of an appropriate size and shape, the article should be completely immersed within the culture medium. Alternatively, the article, if it can be cut or disassembled, should be divided into smaller proportions to allow for immersion within the culture medium.

For solid articles that cannot be readily cut into pieces or immersed into the largest permitted volume of culture media (2000 ml), the article can be rinsed three times with suitable volumes of medium. In doing this, it is important to ensure that all parts of the article come into contact with the medium. The entire washing from the article can then be tested using the membrane filtration method. It must be recognised that this is the least sensitive method available and that some microorganisms may still adhere to the surface of the solid or that trapped air may prevent the medium from reaching all parts of the article. This step is difficult to validate or to demonstrate with any certainty, given the complexities of microbial attachment to surfaces.

Transfusion or Infusion Assemblies

For transfusion or infusion assemblies, or where the size of an item renders immersion impracticable, an alternative approach is to flush the device with a sufficient quantity of fluid thioglycolate medium in order to reach all parts of the item. 20 items are typically tested. The exercise should be repeated with tryptone soya broth using a separate 20 items. The rinse solution of both media should not be less than 15 ml for each item, and the collected total should be a total of not less than 100ml. The media re-incubated as per the standard sterility test criteria.

For some devices, the lumen is so small that fluid thioglycolate medium will not pass through. Here, an alternative thioglycolate medium can be used, provided the alternative medium passes the test for growth promotion.



Bacterial streak (Image: Tim Sandle)

Summary

Given the multitude of different types of sterile products on the marketplace, this paper has considered some general, practical examples for the sterility testing of ‘difficult’ products. In doing so, the focus has been with common product types and widely used techniques.

For products not reviewed in this paper, a framework for considering the appropriate steps to take when developing an alternative method, such as attempting membrane filtration first, exploring rinsing and neutralisation second and so forth, has been provided.

Dealing with difficult products fits into the key steps required for introducing a new product to be sterility tested, which are[32]:

- Selecting culture media
- Performing bacteriostasis / fungistasis testing (method suitability)
- Eliminating any bacteriostatis / fungistatic properties
- Determining the number of articles to test
- Incubate the samples
- Examining test articles for signs of growth
- Examining suspect tubes microscopically for signs of growth
- Subculturing if necessary (for turbid samples)
- Documenting the outcome

The underlying message is that any alternative method must be carefully planned and validated before being adopted and put into routine use.

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About the author

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Dr. Tim Sandle has over twenty-five years' experience of microbiological research and biopharmaceutical processing. He is a member of several editorial boards and he has written over six hundred book chapters, peer reviewed papers and technical articles relating to microbiology. Dr. Sandle works for a pharmaceutical manufacturer in the UK, and is a visiting tutor at both the University of Manchester and UCL.

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