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Traditional Chinese medicine — Determination of microorganisms in natural products

Médecine traditionnelle chinoise — Détermination des micro-organismes dans les produits naturels

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ISO copyright office
CP 401 • Ch. de Blandonnet 8
CH-1214 Vernier, Geneva
Phone: +41 22 749 01 11
Email: copyright@iso.org
Website: www.iso.org

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Foreword

ISO (the International Organization for Standardization) is a worldwide federation of national standards bodies (ISO member bodies). The work of preparing International Standards is normally carried out through ISO technical committees. Each member body interested in a subject for which a technical committee has been established has the right to be represented on that committee. International organizations, governmental and non-governmental, in liaison with ISO, also take part in the work. ISO collaborates closely with the International Electrotechnical Commission (IEC) on all matters of electrotechnical standardization.

The procedures used to develop this document and those intended for its further maintenance are described in the ISO/IEC Directives, Part 1. In particular, the different approval criteria needed for the different types of ISO documents should be noted. This document was drafted in accordance with the editorial rules of the ISO/IEC Directives, Part 2 (see www.iso.org/directives).

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This document was prepared by Technical Committee ISO/TC 249, *Traditional Chinese medicine*.

Any feedback or questions on this document should be directed to the user's national standards body. A complete listing of these bodies can be found at www.iso.org/members.html.

Introduction

Natural products used in traditional Chinese medicine are widely used around the world due to their high medicinal values and mild side effects. It is a common phenomenon that natural products are contaminated by microorganisms which not only impact their quality and efficacy, but also restrict the international trade in them and related products. Although the Pharmacopeia of the People's Republic of China, the British Pharmacopoeia, the Japanese Pharmacopoeia, the European Pharmacopoeia and the United States Pharmacopeia have stipulated the microbial limits of natural products, there is no International Standard for microorganism detection methods, which adversely affects communication and trade between researchers and factories in different countries. Furthermore, microorganism levels on or in natural products usually exceed the maximum limit levels set by many international organizations and countries due to the lack of an International Standard.

Traditional Chinese medicine — Determination of microorganisms in natural products

1 Scope

This document specifies test methods to determine microorganisms in natural products. It is applicable only to natural products used in traditional Chinese medicine, including raw materials, herbal pieces and preparations.

2 Normative reference

There are no normative references in this document.

3 Terms and definitions

For the purposes of this document, the following terms and definitions apply.

ISO and IEC maintain terminological databases for use in standardization at the following addresses:

— ISO Online browsing platform: available at https://www.iso.org/obp

— IEC Electropedia: available at http://www.electropedia.org/

3.1
sterility
state of being free from viable microorganisms

Note 1 to entry: In practice, no such absolute statement regarding the absence of microorganisms can be proven.

[SOURCE: ISO 11139:2018, 3.274]

3.2
microbial enumeration test
quantitative counting of mesophilic bacteria and fungi which may grow under aerobic conditions

4 Symbols and abbreviated terms

ATCC	American Type Culture Collection
CMCC	National Center for Medical Culture Collections
CIP	Collection de Bactéries de I'Institut Pasteur
IMI	International Mycological Institute
IP	Institut Pasteur
MPN	most-probable-number
NBRC	Biological Resource Center, National Institute of Technology and Evaluation
NCIMB	National Collection of Industrial and Marine Bacteria Ltd

NCPF National Collection of Pathogenic Fungi

NCTC National Collection of Type Cultures

TAMC total aerobic microbial count

TYMC total combined yeast and mould count

5 Test methods

5.1 General

The test shall be carried out under aseptic conditions. In order to achieve such conditions, the test environment shall be adapted to the way in which the sterility test is performed. The precautions taken to avoid contamination shall 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 shall be monitored regularly by appropriate sampling of the working area and by carrying out appropriate controls.

5.2 Strains

The standardized stable suspensions of test strains as stated in Table 1 shall be used. Seed-lot culture maintenance techniques (seed-lot systems) shall be used so that the viable microorganisms used for inoculation are not more than five passages removed from the original master seed-lot.

Table 1 — Standard strains

Escherichia coli	ATCC 8739, NCIMB 8545, CIP 53.126 NBRC 3972 or CMCC (B) 44102
Pseudomonas aeruginosa	ATCC 9027, NCIMB 8626, CIP 82.118, NBRC 13275 or CMCC (B) 10104
Clostridium sporogenes	CMCC (B) 64941 or ATCC 11437 (NBRC 14293, NCIMB 12343, CIP 100651) or ATCC 19404 (NCTC 532 or CIP 79.03) or NBRC 14293
Staphylococcus Aureus	ATCC6538, NCIMB9518, CIP 4.83, NBRC 13276 or CMCC (B)26003
Bacillus subtilis	ATCC 6633, NCIMB 8054, CIP 52.62, NBRC 3134 or CMCC (B) 63501
Candida albicans	ATCC 10231, NCPF 3179, IP 48.72 or NBRC 1594 or CMCC (F) 98001
Aspergillus brasiliensis (Aspergillus niger)	ATCC 16404, IMI 149007, IP 1431.83 NBRC 9455 or CMCC (F) 98003
Salmonella paratyphi B	CMCC(B)50094, ATCC 14028 or, as an alternative, *Salmonella enteric* subsp. *enteric* serovar Abony such as NBRC 100797, NCTC 6017 or CIP 80.39
Shigella dysenteriae type 1	ATCC 11835, ATCC 9361, CMCC 51252

5.3 Test for sterility

5.3.1 General

The test is applied to raw materials, herbal pieces and preparations which are required to be sterile. However, a satisfactory result only indicates that no contaminating microorganism has been found in the sample examined under the conditions of the test. The acceptance criteria for microbiological quality of products to be tested shall be done according to A.1 in Annex A.

5.3.2 Culture media and incubation temperatures

Recommended media for the test may be prepared as shown in Annex B, or equivalent commercial media may be used provided that they conform to the growth promotion test.

The culture media have been found to be suitable for the test for sterility. Fluid thioglycollate medium is primarily intended for the culture of anaerobic bacteria. However, it can also detect aerobic bacteria. Soya-bean casein digest medium is suitable for the culture of both fungi and aerobic bacteria.

Fluid thioglycollate medium shall be incubated at 30 °C to 35 °C. For products containing a mercurial preservative that cannot be tested by the membrane-filtration method, fluid thioglycollate medium incubated at 20 °C to 25 °C may be used instead of soya-bean casein digest medium provided that it has been validated as described in the growth promotion test.

5.3.3 Growth promotion test for aerobes, anaerobes and fungi

Test each lot of ready-prepared medium and each batch of medium prepared either from dehydrated medium or from ingredients. Suitable strains of microorganisms are indicated in Table 2.

Inoculate portions of fluid thioglycollate medium with a small number (not more than 100 cfu) of microorganisms, using a separate portion of medium for each of the following species of microorganism: *Clostridium sporogenes*, *Pseudomonas aeruginosa* and *Staphylococcus aureus*.

Inoculate portions of alternative thioglycollate medium with a small number (not more than 100 cfu) of *Clostridium sporogenes*. Inoculate portions of soya-bean casein digest medium with a small number (not more than 100 cfu) of microorganisms, using a separate portion of medium for each of the following species of microorganism: *Aspergillus brasiliensis*, *Bacillus subtilis,* and *Candida albicans*. Incubate for not more than 3 days in the case of bacteria and not more than 5 days in the case of fungi. Seed lot culture maintenance techniques (seed-lot systems) are used so that the viable microorganisms used for inoculation are not more than five passages removed from the original master seed-lot.

Table 2 — Strains of the test microorganisms suitable for use in the growth promotion test and the method validation

Aerobic bacteria	*Staphylococcus aureus* ATCC 6538, CIP 4.83, NCTC 10788, NCIMB 9518, NBRC 13276 or CMCC (B)26003
	Bacillus subtilis ATCC 6633, CIP 52.62, NCIMB 8054, NBRC 3134 or CMCC (B) 63501
	Pseudomonas aeruginosa[a] ATCC 9027, NCIMB 8626, CIP 82.118, NBRC 13275 or CMCC (B) 10104
Anaerobic bacterium	*Clostridium sporogenes*[b] ATCC 19404, CIP 79.3, NCTC 532, ATCC 11437, NBRC 14293 or CMCC (B) 64941
Fungi	*Candida albicans* ATCC 10231, IP 48.72, NCPF 3179, NBRC 1594 or CMCC (F) 98001
	Aspergillus brasiliensis ATCC 16404, IP 1431.83, IMI 149007, NBRC 9455 or CMCC (F) 98003

[a] An alternative microorganism in Aerobic bacteria is *Micrococcus luteus* (ATCC 9341).

[b] An alternative to *Clostridium sporogenes*, when a nonspore-forming microorganism is desired, is *Bacteroides vulgatus* (ATCC 8482).

5.3.4 Method suitability test

5.3.4.1 Membrane filtration

After transferring the contents of the container or containers to be tested to the membrane, add an inoculum of a small number of viable microorganisms (not more than 100 cfu) to the final portion of sterile diluent used to rinse the filter.

5.3.4.2 Direct inoculation

After transferring the contents of the container or containers to be tested to the culture medium, add an inoculum of a small number of viable microorganisms (not more than 100 cfu) to the medium.

In both cases, use the same microorganisms as those described in 5.3.3. Perform a growth promotion test as a positive control. Incubate all the containers containing medium for not more than 5 days.

5.3.5 Test for sterility of the product to be examined

5.3.5.1 General

Unless otherwise specified elsewhere in this document or in the individual monograph, test the number of articles specified in Table 3. If the contents of each article are of sufficient quantity (see Table 3), they may be divided so that equal appropriate portions are added to each of the specified media. (Perform sterility testing employing two or more of the specified media.) If neither article contains sufficient quantities for each medium, use twice the number of articles indicated in Table 4.

The test may be carried out using the technique of membrane filtration or by direct inoculation of the culture medium with the product to be examined. Appropriate negative controls are included. The technique of membrane filtration is used whenever the nature of the product permits; that is, for filterable aqueous preparations, for alcoholic or oily preparations, and for preparations miscible with, or soluble in, aqueous or oily solvents, provided these solvents do not have an antimicrobial effect in the conditions of the test.

5.3.5.2 Membrane filtration

5.3.5.2.1 General

Use membrane filters having a nominal pore size not greater than 0,45 µm, in which the effectiveness to retain microorganisms has been established. Cellulose nitrate filters, for example, are used for aqueous, oily and weakly alcoholic solutions. Cellulose acetate filters, for example, are used for strongly alcoholic solutions. Specially adapted filters may be needed for certain products.

Sterility test assumes that membranes about 50 mm in diameter will be used. If filters of a different diameter are used, the volumes of the dilutions and the washings should be adjusted accordingly. The filtration apparatus and membrane shall be sterilized by appropriate means. The apparatus is designed so that the solution to be examined can be introduced and filtered under aseptic conditions. It permits the aseptic removal of the membrane for transfer to the medium or it is suitable for carrying out the incubation after adding the medium to the apparatus itself.

5.3.5.2.2 Aqueous solutions

If appropriate, transfer a small quantity of a suitable, sterile diluent such as a 1 g/l neutral solution of meat or casein peptone pH 7,1 ± 0,2 onto the membrane in the apparatus and filter. The diluents may contain suitable neutralizing substances, appropriate inactivating substances or both, for example in the case of antibiotics.

Transfer the contents of the container or containers to be tested to the membrane or membranes, if necessary, after diluting to the volume used in 5.3.4 with the chosen sterile diluent; in any case, using not less than the quantities of the product to be examined prescribed in Table 3. Filter immediately. If the product has antimicrobial properties, wash the membrane not less than three times by filtering through it each time the volume of the chosen sterile diluent used in 5.3.4. Do not exceed a washing cycle of five times 100 ml per filter, even if during the method suitability test it has been demonstrated that such a cycle does not fully eliminate the antimicrobial activity. Transfer the whole membrane to the culture medium or cut it aseptically into two equal parts and transfer one half to each of two suitable media. Use the same volume of each medium as in the method suitability test. Alternatively, transfer the medium onto the membrane in the apparatus. Incubate the media for not less than 14 days.

5.3.5.2.3 Soluble solids

Use for each medium not less than the quantity prescribed in Table 3 of the product dissolved in a suitable solvent, such as the solvent provided with the preparation, water for injections, saline or a 1 g/l neutral solution of meat or casein peptone. Proceed with the test as described in 5.3.5.2.2 or aqueous solutions using a membrane appropriate to the chosen solvent.

5.3.5.2.4 Oils and oily solutions

Use for each medium not less than the quantity of the product prescribed in Table 3. Oils and oily solutions of sufficiently low viscosity may be filtered without dilution through a dry membrane. 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. Allow the oil to penetrate the membrane by its own weight then filter, applying the pressure or suction gradually. Wash the membrane at least three times by filtering through it each time about 100 ml of a suitable sterile solution, such as 1 g/l neutral meat or casein peptone containing a suitable emulsifying agent at a concentration shown to be appropriate in 5.3.4, for example polysorbate 80 at a concentration of 10 g/l. Transfer the membrane or membranes to the culture medium or media or vice versa as described in 5.3.5.2.2, and incubate at the same temperature for the same time.

5.3.5.2.5 Ointments and creams

Use for each medium not less than the quantities of the product prescribed in Table 3. Ointments in a fatty base and emulsions of the water-in-oil type may be diluted to 1 % in isopropyl myristate as described in 5.3.5.2.4 by heating, if necessary, to not more than 40 °C. In exceptional cases it may be necessary to heat to not more than 44 °C. Filter as rapidly as possible and proceed as described in 5.3.5.2.4.

5.3.5.2.6 Sterile aerosol products

For fluid products in pressurized aerosol form, freeze the containers in an alcohol-dry ice mixture of at least –20 °C for about 1 hour. If feasible, allow the propellant to escape before aseptically opening the container and transfer the contents to a sterile pooling vessel. Add 100 ml of fluid D (see Table B.2) to the pooling vessel and mix gently. Proceed as described in 5.3.5.2.2 or 5.3.5.2.4, whichever applies.

Table 3 — Minimum quantity to be used for each medium

Quantity per container	Minimum quantity to be used for each medium unless otherwise justified and authorized
Liquids	
— less than 1 ml	The entire contents of each container
— 1 ml to 40 ml	Half the contents of each container but not less than 1 ml
— greater than 40 ml and less than 100 ml	20 ml
— greater than 100 ml	10 % of the contents of the container but not less than 20 ml
Antibiotic liquids	1 ml
Insoluble preparations, creams and ointments to be suspended or emulsified	Use the contents of each container to provide not less than 200 mg
Solids	
Less than 50 mg	The entire contents of each container
50 mg or more but less than 300 mg	Half the contents of each container but not less than 50 mg
300 mg to 5 g	150 mg
Greater than 5 g	500 mg

Table 4 — Minimum number of items to be tested

Number of items in the batch	Minimum number of items to be tested for each medium, unless otherwise justified and authorized
Parenteral preparations	
Not more than 100 containers	10 % or four containers, whichever is greater
More than 100 but not more than 500 containers	10 containers
More than 500 containers	2 % or 20 containers (10 containers for large-volume parenterals), whichever is less
Ophthalmic and other non-injectable	
Not more than 200 containers	5 % or two containers, whichever is greater
More than 200 containers	10 containers
If the product is presented in the form of single-dose containers, apply the scheme shown for preparations for parenteral use	
Bulk solid products	
Up to four containers	Each container
More than four containers but not more than 50 containers	20 % or four containers, whichever is greater
More than 50 containers	2 % or 10 containers, whichever is greater

5.3.5.3 Direct inoculation of the culture medium

5.3.5.3.1 General

Transfer the quantity of the preparation to be examined prescribed in Table 3 directly into the culture medium so that the volume of the product is not more than 10 % of the volume of the medium, unless otherwise prescribed.

If the product to be examined has antimicrobial activity, carry out the test after neutralizing this with a suitable neutralizing substance or by dilution in a sufficient quantity of culture medium. When it is necessary to use a large volume of the product, it may be preferable to use a concentrated culture medium prepared in such a way that it takes account of the subsequent dilution. Where appropriate, the concentrated medium may be added directly to the product in its container.

5.3.5.3.2 Oily liquids

Use media to which have been added a suitable emulsifying agent at a concentration shown to be appropriate in the method suitability test, for example polysorbate 80 at a concentration of 10 g/l.

5.3.5.3.3 Ointments and creams

Prepare by diluting to about 1 in 10 by emulsifying with the chosen emulsifying agent in a suitable sterile diluent, such as a 1 g/l neutral solution of meat or casein peptone. Transfer the diluted product to a medium not containing an emulsifying agent.

Incubate the inoculated media for not less than 14 days. Observe the cultures several times during the incubation period. Shake cultures containing oily products gently each day. However, when fluid thioglycollate medium is used for the detection of anaerobic microorganisms, keep shaking or mixing to a minimum in order to maintain anaerobic conditions.

5.3.5.4 Observation and interpretation of results

At intervals during the incubation period and at its conclusion, examine the media for macroscopic evidence of microbial growth. If the material being tested renders the medium turbid so that the presence or absence of microbial growth cannot be readily determined by visual examination, 14 days

after the beginning of incubation 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 validity of the test is established according to 6.1.4. If the test is declared to be invalid, it is repeated with the same number of units as in the original test.

5.3.5.5 Minimum number of items to be tested

The minimum number of items to be tested in relation to the size of the batch is given in Table 4. If the batch size is unknown, use the maximum number of items prescribed. If the contents of one container are enough to inoculate the two media, this column gives the number of containers needed for both the media together.

5.4 Microbiological examination of non-sterile products: microbial enumeration tests

5.4.1 General

The test is applied to determinate whether the non-sterile products involving raw materials, herbal pieces and preparations conformed to the standard or not.

If the product to be examined has antimicrobial activity, this is as far as possible removed or neutralized. If neutralizers or inactivators are used in tests, their efficacy and their absence of toxicity for microorganisms shall be demonstrated. If surfactants are used for sample preparation, their absence of toxicity for microorganisms and their compatibility with any neutralizers or inactivators used shall be demonstrated.

The enumeration methods involve membrane filtration, the plate count method and the most-probable-number (MPN) method. Use the membrane filtration method or one of the plate-count methods, as directed. The MPN method is generally the least-accurate method for microbial counts; however, for certain product groups with very low bioburden it might be the most appropriate method.

The choice of a method is based on factors such as the nature of the product and the required limit of microorganisms. The method chosen shall allow testing of a sufficient sample size to judge conformity with the specification. The suitability of the chosen method shall be established.

The acceptance criteria for microbiological quality of products to be tested shall be done according to A.2 (Table A.1 to Table A.15) in Annex A.

5.4.2 Growth promotion test, suitability of the counting method and negative controls

5.4.2.1 General

The ability of the test to detect microorganisms in the product to be tested shall be established. Suitability shall be confirmed if a change in testing performance or a change in the product that may affect the outcome of the test is introduced.

5.4.2.2 Preparation of test strains

Use standardized stable suspensions of test strains or prepare as described in Table 5. Seed-lot culture maintenance techniques (seed-lot systems) are used so that the viable microorganisms used for inoculation are not more than five passages removed from the original master seed-lot. Grow each of the bacterial and fungal test strains separately as described in Table 5.