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Footwear and footwear components — Qualitative test method to assess antifungal activity (growth test)

Chaussures et composants de chaussures — Méthode d'essai qualitative pour évaluer l'activité antifongique (essai de croissance)

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Foreword

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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).

Attention is drawn to the possibility that some of the elements of this document may be the subject of patent rights. ISO shall not be held responsible for identifying any or all such patent rights. Details of any patent rights identified during the development of the document will be in the Introduction and/or on the ISO list of patent declarations received (see www.iso.org/patents).

Any trade name used in this document is information given for the convenience of users and does not constitute an endorsement.

For an explanation of the voluntary nature of standards, the meaning of ISO specific terms and expressions related to conformity assessment, as well as information about ISO's adherence to the World Trade Organization (WTO) principles in the Technical Barriers to Trade (TBT), see www.iso.org/iso/foreword.html.

This document was prepared by Technical Committee ISO/TC 216, *Footwear*, in collaboration with the European Committee for Standardization (CEN) Technical Committee CEN/TC 309, *Footwear,* in accordance with the Agreement on technical cooperation between ISO and CEN (Vienna Agreement).

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.

Footwear and footwear components — Qualitative test method to assess antifungal activity (growth test)

CAUTION — Test methods specified herein require the use of micro-fungi. These tests shall only be carried out in facilities with containment techniques for handling microorganisms and by persons with training and experience in the use of microbiological techniques.

1 Scope

This document specifies a test method (growth test) for the qualitative evaluation of the antifungal activity of footwear and footwear components exposed to the action of filamentous micro-fungi.

This document is only applicable to footwear and components that claim to have antifungal (antimycotic) or antimicrobial treatment effects.

2 Normative references

The following documents are referred to in the text in such a way that some or all of their content constitutes requirements of this document. For dated references, only the edition cited applies. For undated references, the latest edition of the referenced document (including any amendments) applies.

ISO 7218, *Microbiology of food and animal feeding stuffs — General requirements and guidance for microbiological examinations*

ISO 11133, *Microbiology of food, animal feed and water — Preparation, production, storage and performance testing of culture media*

ISO 16187, *Footwear and footwear components — Test method to assess antibacterial activity*

ISO 19952, *Footwear — Vocabulary*

3 Terms and definitions

For the purposes of this document, the terms and definitions given in ISO 19952 and the following apply.

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

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

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

3.1
antifungal activity
antimycotic activity
efficacy of a material or finish used to prevent or mitigate the growth of micro-fungi, to reduce the number of micro-fungi or to kill micro-fungi

3.2
control specimen
material identical to the test material but without antifungal treatment

4 Principle

The test and control specimens of footwear are inoculated with a mixed spore suspension of selected mould test strains or with a single test strain according to the specific claim.

Antifungal performance is qualitatively determined by visual assessment of fungal growth after specified or agreed incubation period.

5 Safety

Handling of microorganisms that are potentially hazardous requires a high degree of technical competence and can be subject to current national legislation and regulations. Only personnel trained in microbiological techniques should carry out such tests.

NOTE Refer to country-specific codes of practice for personal hygiene, disinfection and sterilization.

The persons who perform the test should consult IEC 60068-2-10:2005+AMD1:2018, Annex A and ISO 7218.

6 Apparatus

6.1 General

Disposable apparatus is an acceptable alternative to re-usable glassware and plastic if it has suitable specifications.

The usual microbiological laboratory equipment in accordance with ISO 7218 and in particular the following shall be used.

6.2 Biological safety cabinet.

6.3 Microbiological incubator, capable of maintaining a temperature of (28 ± 2) °C and a relative humidity of (85 ± 5) %.

6.4 Autoclave, capable of maintaining a temperature of (121 ± 2) °C and a pressure of (103 ± 5) kPa, for wet sterilization, used in accordance with ISO 7218.

6.5 Vortex mixer.

6.6 pH-meter, having an accuracy of ±0,1 pH-units.

6.7 Laboratory centrifuge, 2 000 g.

NOTE 2 000 g ≈ 4 000 r/min.

6.8 Microscope, at least magnification x 20 (better magnification x 50).

6.9 Glass beads, 2 mm to 3 mm in diameter, for preparation of fungal spore suspension.

6.10 Glass wool or medical gauze (double layers), for preparation of fungal spore suspension.

6.11 Wide mouth jars, with cap, 500 ml, capable of being used with an autoclave (6.4).

6.12 Oven, for dry sterilization.

6.13 Balance, capable of weighing to ±0,01 g.

6.14 Spectrophotometer, capable of measuring at a 500 nm to 700 nm wavelength, or McFarland's nephelometer.

6.15 Petri dishes, that have been sterilized, made of glass or plastic, in diameter sizes of 90 mm to 100 mm or 55 mm to 60 mm.

6.16 Pipettes, having the most suitable volume for each use.

6.17 Grid, for evaluation of fungal growth, each square has a size of 5 mm × 5 mm, or that divides the surface of the test specimen into 100 equal sized squares.

7 Reagents and culture medium

7.1 General

The reagents used in the tests shall be of analytical quality and/or suited for microbiological purposes.

Dehydrated products available on the commercial market should be used in the preparation of the culture media. The manufacturer's instructions for the preparation of these products should be strictly followed.

The preparation, production and performance testing of culture media shall be in accordance with ISO 11133.

7.2 Water

The water used in the tests shall be analytical-grade water for microbiological media preparation, which is freshly distilled and/or ion-exchanged and/or ultra-filtered and/or filtered with RO (Reverse Osmosis).

It shall be free from all toxic or microorganism inhibitory substances.

7.3 Malt Extract Agar (MEA) medium

7.3.1 Composition

Malt extract	30,0 g
Soya peptone	3,0 g
Agar	15,0 g
Water	1 000 ml

7.3.2 Preparation

After mixing, stir and adjust the pH to (5,5 ± 0,2) at room temperature. Heat with stirring on a hotplate or in a boiling-water bath until the components are completely dissolved, sterilize at (121 ± 2) °C for 15 min in an autoclave (6.4) with saturated water vapour. Cool and shake solution well, then pour 25 ml into each sterile Petri dish. Leave to cool and solidify.

NOTE 1 The Potato Dextrose Agar (PDA) can also provide a complete medium for the growth of micro-fungi. The PDA medium with standard composition can be obtained from commercial source.

NOTE 2 MEA medium can be obtained from commercial source.

7.4 Physiological saline (sodium chloride solution)

7.4.1 Composition

Sodium chloride (NaCl), CAS RN®[a]: 7647-14-5	8,5 g
Water	1 000 ml

[a] CAS Registry Number® (CAS RN®) is a trademark of CAS corporation. This information is given for the convenience of users of this document and does not constitute an endorsement by ISO of the product named. Equivalent products may be used if they can be shown to lead to the same results.

7.4.2 Preparation

After mixing well, adjust pH to (6,9 ± 0,2) at room temperature and sterilize at (121 ± 2) °C for 15 min.

7.5 Wetting agent (nonionic surfactant)

The wetting agent shall be used to harvest the spores and in the test spore suspension. It should not react with other reagents and not cause a reduction or increase in micro-fungi number, such as polysorbate 80 (TWEEN 80), N-methyltauride, Triton™ X-100[1)] or polyglycol ether, etc. Use final concentrations of 0,01 %.

NOTE Wetting agent (nonionic surfactant) can be used in the test when the specimens have coating.

7.6 Buffer solution

7.6.1 Buffer stock

Potassium dihydrogen phosphate (KH_2PO_4), CAS RN®: 7778-77-0	34,0 g
Water	1 000 ml

7.6.2 Preparation of buffer stock

Weigh potassium dihydrogen phosphate into 1 000 ml flask, add 500 ml of distilled water, adjust pH to (7,2 ± 0,2) (at room temperature) with diluted solution of 0,01 mol/l NaOH. Dilute to 1 000 ml with distilled water and store at 4 °C for maximum 6 month.

7.6.3 Preparation of buffer solution

Transfer 1 ml of buffer stock solution and 0,08 g of wetting agent (7.5), corresponds to 0,01 % and dilute to 800 ml with distilled water. After mixing well, sterilize at (121 ± 2) °C for 15 min.

NOTE The wetting agent (7.5) can be omitted if not required.

8 Test microorganisms

The strain used shall be stated in the test report.

The species that shall be used are listed in Table 1.

1) Triton™ X-100 is the trade name of a product supplied by SIGMA-ALDRICH. This information is given for the convenience of users of this document and does not constitute an endorsement by ISO of the product named. Equivalent products may be used if they can be shown to lead to the same results.

If antimycotic properties are claimed against mould, tests can be carried out against one of *Aspergillus sp*. and *Penicillium funiculosum*, either as a mixed suspension or separately. If antimycotic properties are claimed against dermatophyte athlete's foot, *Trichophyton mentagrophytes* shall be used for testing.

Do not test a mixed suspension of mould strains and dermatophyte strains.

Each microorganism should be tested independently.

Strains can be preserved in accordance with the supplier's instructions or EN 12353.

Table 1 — Test strains

Micro-fungi[a]	Name	WDCM No.	CGMCC No.	ATCC® No.[c]
Mould/ Aspergillus sp.[b]	*Aspergillus niger* *or* *Aspergillus brasiliensis*	00144 or 00053	CGMCC 3.4463 or CGMCC 3.5487	ATCC® 6275™ or ATCC® 16404™
Mould/ Penicillium sp.	*Penicillium funiculosum*	00194	CGMCC 3.3875	ATCC® 9644™
Dermatophyte	*Trichophyton mentagrophytes*	00191	—	ATCC® 9533™

Key

WDCM: World Data Centre for Microorganisms

CGMCC: China General Microbiological Culture Collection Centre

ATCC®: American Type Culture Collection

NOTE Other Micro-fungi (appropriate species or other appropriate strains) can be used after appropriate validation.

[a] Test strains shall be obtained from agencies of the World Federation of Culture Collection (WFCC). The micro-fungal species and their supply sources shall be stated in the test report.

[b] Choose one of the *Aspergillus* strains (either *A. niger* or *A. brasiliensis*).

[c] ATTC is a trademark of American Type Culture Collection. ATCC® 6275™, ATCC® 16404™, ATCC® 9644™ and ATCC® 9533™ are examples of suitable products available commercially. This information is given for the convenience of users of this document and does not constitute an endorsement by ISO of these products.

9 Preparation of test spore suspension

9.1 Apply strains within 4 generations.

9.2 Inoculate the micro-fungi spores onto the MEA medium (7.3) surface or standard PDA medium surface, and incubate at 28 °C ± 2 °C until the surface is full of micro-fungi spores (about 1 week to 2 weeks for *Aspergillus sp./Penicillium sp.* and 2 weeks to 3 weeks for *Trichophyton sp.*).

9.3 In each culture tube or plate, place 5 ml of physiological saline (7.4), and a non-toxic wetting agent (7.5) that may be added at a final concentration of 0,01 %. Gently scrape the surface of the sporulating culture with a sterile inoculation loop and introduce them into the culture tube or plate to obtain an aqueous suspension of the spores. Gently shake the culture tube or plate to disperse the spores in the liquid. Wash out spores and pour into a 125 ml sterile conical flask containing 10 sterile glass beads to 15 sterile glass beads (6.9).

Repeat this procedure with the same culture plate twice. Shake the conical flask in order to get full uniform spore suspension. The spore suspension of each fungal culture is filtered at least twice through four thin layers of medical gauze or glass wool (6.10) to remove mycelia fragments, agar blocks and to separate combined spores.

Aseptically centrifuge the filtered spore suspension under a relative centrifugal force of 2 000 g for 1 min and discard the upper liquid. Re-suspend the residue in 20 ml physiological saline (7.4), and

centrifuge again. Repeat washing of the spores for at least three times with this method and check by microscopy to ensure absence of mycelia fragments.

Dilute spore suspensions with buffer solution (7.6), adjust the concentration to 1,0 × 10^6 spores per ml to 5,0 × 10^6 spores per ml, as determined with counting chamber. Other appropriate methods could also be applied in the determination of spore concentration.

9.4 Repeat these operations with each test micro-fungus. For testing of moulds, when using a mixed spore suspension, blend equal volumes of one *Aspergillus sp*. suspension and *Penicillium sp*. suspension containing the same number of spores to obtain the final mixed spore suspension ready for inoculation. Use fresh spore suspension, or spore suspension stored in a fridge at 2 °C to 8 °C within 4 days after preparation.

Do not blend *Trichophyton mentagrophytes* suspension with any other micro-fungi suspension.

10 Preparation of test specimens

10.1 General

Test only the components or materials that are claimed to be antifungal. If the whole footwear is claimed to be antifungal, major components, including upper, lining, insole, insock, outsole shall be tested separately.

In the case where only one material of a component is claimed to be antifungal, it shall be tested separately, if possible. Otherwise, the whole component shall be tested.

Each test specimens shall be at least 80 % of the surface area of the component or material. If a single material accounts for less than 80 %, take the two main materials used in the composition of the component.

The test specimens can be obtained directly from the footwear or from components and materials prior to construction.

10.2 Test and control specimens

The area of test specimens should be about 1 600 mm^2 for testing, and have a thickness of less than 5,0 mm. The area and the weight shall be reported in the test report. If a larger test specimen is used, then the volume of micro-fungal suspension should be increased proportionally.

If it is impossible to lower the thickness of the test specimens (for example, components are thicker and can't be separated or cut without changing critical properties, such surface morphology, which can affect how the fungi interact with the surface), the thickness shall be indicated in the test report.

Use material identical to the test material but without antifungal treatment as control specimens. If no such material is available, sterilized filter paper can be used as control specimens.

At least three test specimens shall be taken for each material or component and three control specimens for each test.

10.3 Pre-treatment of test and control specimens

Pre-treatment of test and control specimens is optional and should only be conducted if necessary due to high bioburden (contamination etc.).