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Footwear and footwear components — Quantitative challenge test method to assess antifungal activity

Chaussures et composants de chaussure — Méthode de test d'épreuve quantitatif pour évaluer l'activité antifongique

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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).

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This document was prepared by Technical Committee ISO/TC 216, *Footwear*.

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 — Quantitative challenge test method to assess antifungal activity

CAUTION — Test methods specified herein require the use of micro-fungi. These tests are only to 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 quantitative challenge test methods for evaluating the antifungal activity of footwear and footwear components.

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

Two methods can be applied. The choice of method depends on the material properties and test microorganisms. Dynamic challenge test method can be applied to all types of materials. For single absorbent materials, static challenge test method is recommended. Brief descriptions of each method are given in 11.2 and 11.3.

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 19952, *Footwear — Vocabulary*

3 Terms and definitions

For the purpose of this document, the terms and definitions given in ISO 19952 and the following 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
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

Note 1 to entry: If no control specimen is available, sterilized conical flask can be used as control specimen.

3.3
neutralizer
chemical agents used to inactivate, neutralize, or quench the antifungal properties of antifungal agents

[SOURCE: ISO 20743:2013, 3.7, modified — "antibacterial" has been replaced with "antifungal".]

4 Principle

The test specimens and control specimens are inoculated with a spore suspension of a selected test strain of micro-fungi specified or claimed. Two test methods are available to assess antifungal activity.

Antifungal performance is quantitatively determined by counting the number of viable micro-fungi and calculating the antifungal activity ratio.

5 Safety

The handling of microorganisms which 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.

It is recommended that the person who perform the test should consult IEC 60068-2-10:2005, Appendix 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.

Usual microbiological laboratory equipment in accordance with ISO 7218 and in particular the following.

6.2 Biological safety cabinet.

6.3 Incubator, capable of maintaining a temperature of (28 ± 2) °C.

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

6.6 Ultraviolet lamp.

6.7 Wide mouth jars, with cap, 100 ml, capable of being used with an autoclave (6.4).

6.8 Vortex mixer.

6.9 Centrifugal machine, 2 000 × *g*.

6.10 Dimensional shaker, two dimensional or three dimensional, capable of adjusting to 50 r/min.

6.11 Shaking incubator, capable of maintaining a temperature of (28 ± 2) °C and a rotational frequency of (120 ± 10) r/min.

6.12 Glass beads, 2 mm to 3 mm in diameter, 10 beads to 15 beads per conical flask, for preparation of fungal spore solutions.

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

6.14 Oven, for dry sterilization.

6.15 pH-meter, capable of measuring to ±0,2 units.

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

6.17 Spectrophotometer, capable of measuring at 500 nm to 660 nm wavelength, or McFarland's nephelometer.

6.18 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.

6.19 Pipette, having the most suitable volume for each use.

7 Reagents and culture medium

7.1 General

The preparation and test shall be freshly prepared in order to ensure the culture quality.

NOTE This could be done according to ISO 11133, or according to national standards or regulations.

Reagents used in tests shall be of analytical grade and/or suited for microbiological purposes.

7.2 Water

Water used in 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 reverse osmosis.

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

7.3 Malt medium

7.3.1 Composition

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

7.3.2 Preparation

Dissolve the designated amounts of components in distilled water, stir and adjust pH to (5,5 ± 0,2) at room temperature, sterilize at (121 ± 2) °C for 15 min in an autoclave (6.4) with saturated water vapour.

7.4 Malt extract agar (MEA) medium

7.4.1 Composition

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

7.4.2 Preparation

After mixing, stir and adjust 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 into the Petri dishes.

NOTE The potato dextrose agar (PDA) can also provide a complete medium for the growth of micro-fungi. The standard PDA medium can be obtained as a commercial product thereby avoiding the preparation steps of cooking and the variation in the composition of potato species. The commercial PDA medium with standard composition can be used to avoid the influence of composition of potato and operation when boiling it, and Malt Extract Agar (MEA) medium can be obtained from commercial source.

7.5 Physiological saline (sodium chloride solution)

7.5.1 Composition

Sodium chloride, NaCl	8,5 g
Water	1 000 ml

7.5.2 Preparation

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

7.6 Wetting agent (nonionic surfactant)

To be used for harvesting the spores, does not react with other reagents and does 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 and so on.

NOTE Wetting agent (nonionic surfactant) can be used when the specimen has coating.

7.7 Buffer solution

7.7.1 Buffer stock

Potassium dihydrogen phosphate, KH_2PO_4	34,0 g
Water	1 000 ml

1) TritonTM 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.

7.7.2 Preparation of buffer stock

Prepare phosphate buffer solution by placing 34,0 g of potassium dehydrogenate phosphate in a 1 000 ml volumetric flask. Add 500 ml of water and mix to dissolve. Adjust the pH to 7,2 ± 0,2 at room temperature with sodium hydroxide. Add distilled water to make up to 1 000 ml.

7.7.3 Preparation of buffer solution

Transfer 1 ml of buffer stock solution and 0,08 g of wetting agent (nonionic surfactant) (7.6), corresponds to 0,01 % and dilute to 800 ml with distilled water. After mixing well, sterilize at (121 ± 2) °C for 15 min.

NOTE If wetting agent (nonionic surfactant) is not required, it might be omitted.

8 Test microorganisms

The strain used shall be stated in the test report.

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

Table 1 — Test strains

Micro-fungi[a]	Name	WDCM No.	CGMCC No.	ATCC® No.
Yeast	*Candida albicans*	00054	AS 1.2031	ATCC® 10231™ [b]
Filamentous micro-fungi/Mould	*Aspergillus niger*	00144	AS 3.4463	ATCC® 6275™ [b]
	Aspergillus brasiliensis	00053	AS 3.5487	ATCC® 16404™ [b]
Filamentous micro-fungi/Dermato-phyte	*Trichophyton menta-grophytes*	00191	—	ATCC® 9533 ™ [b]

Key

WDCM: World Data Centre for Microogranisms

CGMCC: China General Microbiological Culture Collection Centre

ATCC®: American Type Culture Collection

NOTE 1 Refer to WDCM and website: http://refs.wdcm.org.

NOTE 2 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 reports.

[b] ATCC® 10231™, ATCC® 6275™, ATCC® 16404™ 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.

Testing of yeast (*Candida albicans*) is mandatory.

If activity against mould is claimed, testing of one mould strain shall be performed.

If activity against dermatophyte is claimed, testing of *Trichophyton mentagrophytes* shall be performed.

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

9 Preparation of test inoculums

9.1 Indications for use of strains

Apply strains within 4 generations.

9.2 Preparation of inoculums of *Candida albicans*

9.2.1 Using a sterile inoculating loop, transfer one *Candida albicans* colony into 20 ml of malt medium (7.3) and incubate in the shaking incubator at (28 ± 2) °C for about 16 h to 20 h (overnight culture), or incubate in the plate of standard PDA medium at (28 ± 2) °C for about 24 h to 48 h.

9.2.2 Prepare fresh buffer solution (7.7) without wetting agent (nonionic surfactant) (7.6), and use this medium to prepare a suspension. Estimate the number of yeast cells with plate count method or spectrophotometer or other appropriate methods, with a yeast concentration of $1{,}0 \times 10^5$ CFU/ml to $5{,}0 \times 10^5$ CFU/ml as test inoculums.

If necessary, store the test inoculums at room temperature and use it within 2 h.

9.3 Preparation of test spore suspension of filamentous micro-fungi

9.3.1 Inoculate the micro-fungi spores to the MEA medium (7.4) (or PDA medium) surface, and incubate at (28 ± 2) °C until the surface is full of micro-fungi spores (about 1 week to 2 weeks for *Aspergillus sp.* and 2 weeks to 3 weeks for *Trichophyton sp*.).

9.3.2 Place in each culture tube or plate 5 ml of physiological saline (7.5). A non-toxic wetting agent (nonionic surfactant) (7.6) may be added with a final concentration of 0,01 %. Gently scrape the surface of the sporulating culture with a sterile inoculation loop 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 sterile conical flask containing sterile glass beads. Repeat this procedure with the same culture tube or 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 double layers of medical gauze or glass wool to remove mycelia fragments, agar blocks and to separate combined spores.

Aseptically centrifuge the filtered spore suspension under the centrifugal force of 2 000 *g* for 1 min, discard the upper liquid. Re-suspend the residue in 20 ml physiological saline (7.5), and centrifuge again. Repeat washing of the spores at least three times with this method. Dilute spore suspensions with buffer solution (7.7). Adjust the concentration to $1{,}0 \times 10^6$ spores per ml to $5{,}0 \times 10^6$ spores per ml, as determined using a counting chamber. Other appropriate methods could also be applied in the determination of spore concentration. Use the fresh spore suspension, or store it in fridge at 2 °C to 8 °C within 3 days after preparation.

NOTE Due to possible lower recovery rate of fungal spores of filamentous micro-fungi, a higher concentration inoculum is indicated with filamentous micro-fungi.

10 Preparation of test specimens

10.1 General

Test only the components or materials which are claimed to be antifungal. If the whole footwear is claimed as 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 specimen shall be at least 80 % of the surface area of the component or material. If single material accounts for less than 80 %, take two main materials used in the composition of the component.

The test specimens can alternatively be obtained directly from the footwear raw materials.

10.2 Test specimen

The area of test specimen should be about 500 mm^2 and have a thickness of less than 5,0 mm. Keep the test specimen in one piece, and do not cut into small pieces. 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 specimen (for example, components are thicker and can't be separated or cut without changing critical properties like surface morphology which may affect how the micro-fungi interact with the surface), the thickness shall be indicated in the test report.

At least 6 test specimens shall be taken for each material or component and for each test strain.

10.3 Pre-treatment of test specimen

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

If sterilization methods are applied, they shall be reported in detail, and shall not affect the antifungal properties or the material itself.

NOTE The test and control specimen can be sterilized by autoclave (6.4) at (121 ± 2) °C and 103 kPa for 15 min, or with ultraviolet rays (ultraviolet lamp 30 W, placed 300 mm away from the specimen, each side for one hour respectively) or other suitable sterilizing methods.

11 Test procedure

11.1 Summary of test methods

Table 2 lists the circumstances under which each test method should be applied. The choice of method depends on the material properties and the test microorganisms.

Table 2 — List of test methods

Method	Dynamic challenge test (11.2)	Static challenge test[b] (11.3)
Type of material[a]	Absorbent and non-absorbent materials or combinations	Absorbent materials[c]
Micro-fungi	Yeast and filamentous micro-fungi	Yeast and filamentous micro-fungi

[a] For the classification of absorbent, non-absorbent, and combined materials, refer to ISO 16187.

[b] For single absorbent materials, static challenge test might be preferred.

[c] Do not include materials that cannot be fully eluted.

11.2 Dynamic challenge tests

11.2.1 Inoculation

Place six test specimens (three for time 0 h and three for time 24 h) and six control specimens (three for time 0 h and three for time 24 h) into sterilized 250 ml conical flasks separately.

Pipette (50 ± 0,5) ml of the inoculum prepared in 9.2 or 9.3 to inoculate each test specimen and control specimen.

If no control specimens are available, inoculate sterile conical flasks without specimens as control to determine the test effectiveness.

11.2.2 Neutralization and elution after inoculation (time 0 h)

After inoculation, tighten caps and shake by vortex mixer for 1 min × 5 cycles or shake by hand in an arc of approximately 30 cm for 1 min × 5 cycles. Then test specimens and control specimens at time 0 h should be neutralized immediately.

Pipette 0,2 ml from three incubated test and control specimens into a test tube with 19,8 ml physiological saline (7.5) and shake them in an arc of approximately 30 cm for 30 s, or mix for 5 s × 5 cycles by vortex mixer (6.8) in order to elute out the micro-fungi into the medium.

Other neutralization solutions (according to EN 1275:2005, Annex B) might be used if appropriate and shall be documented in the test report.

11.2.3 Incubation

Culture the other three inoculated test specimens and the other three inoculated control specimens at (28 ± 2) °C for (24 ± 2) h in a shaking incubator (6.11) with 120 r/min.

11.2.4 Neutralization and elution after incubation (time 24 h)

At time 24 h of incubating of three test specimens and three control specimens (if available), neutralize and elute according to the process as in 11.2.2.

11.2.5 Determination of the number of viable micro-fungi

Take (1,0 ± 0,1) ml elution from 11.2.2 or elution from 11.2.4 with a sterile pipette and add it into a test tube with (9,0 ± 0,1) ml physiological saline (7.5) and shake it well. Dilute the elution with physiological saline (7.5) and get 10-fold serial dilutions.

Inoculate 100 µl of each dilution with a sterile pipette onto MEA medium (7.3) or PDA medium in duplicate, turn the agar upside down and incubate it at (28 ± 2) °C for 48 h for *Candida albicans*, and 3 days to 7 days for *Aspergillus sp.*, and 1 week to 2 weeks for *Trichophyton mentagrophytes*.

After incubation, count the number of colonies in the Petri dishes containing 8 to 300 colonies. If the number of colonies is less than 8, then count and record the number of colonies in these plates. If there are no colonies recovered in the plate, record the number of colonies as <1. If the number of colonies is not to a gradient ratio with the serial dilutions, record and calculate the result based on the larger dilution.

11.3 Static challenge test

11.3.1 Inoculation

Place each of the six test specimens (three for time 0 h and three for time 24 h) and the six control specimens (three for time 0 h and three for time 24 h) into a separate sterilized wide mouth jars.

Pipette (1,0 ± 0,1) ml of the inoculum prepared in 9.2 or 9.3 to each test and control specimen, tightly close the screw cap.

NOTE Because the volume of inoculum has great relationship with test specimens, it could be reduced from 1 ml of inoculum to 100 µl to guarantee the full contact of inoculum with specimen and avoid the spread to the bottle.

If no control specimens are available, inoculate sterile wide mouth jars without specimens as control to determine the test effectiveness.

11.3.2 Neutralization and elution after inoculation (time 0 h)

Three test specimens and three control specimens should be neutralized and eluted immediately after the initial inoculation.

Add 100 ml physiological saline (7.5) to each of three inoculated test and control specimens (if available).

Tighten the caps and shake them in an arc of approximately 30 cm for 30 s, or mix for 5 s × 5 cycles by vortex mixer (6.8) in order to elute out the micro-fungi into the medium.

11.3.3 Incubation

Culture the other three inoculated test specimens and the other three inoculated control specimens at (28 ± 2) °C for (24 ± 2) h.

11.3.4 Neutralization and elution after incubation (time 24 h)

After the incubation at time 24 h, neutralize and elute according to the process as in 11.3.2.

11.3.5 Determination of the number of viable micro-fungi

Determine the number of viable *Candida albicans* of each specimen according to 11.2.5.

12 Expression of results

12.1 Calculation of the number of viable micro-fungi

For each test specimen, determine the number of viable micro-fungi recovered in accordance with Formula (1):

$$M = Z \times B \times 100 \quad (1)$$

where

M is the number of viable micro-fungi of each test specimen (expressed as CFU/ml);

Z is the average number of viable micro-fungi in the two Petri dishes;

B is the dilution ratio;

100 is the dilution ratio of neutralization.

12.2 Judgement of test effectiveness

a) The extreme difference value of the three control specimens after inoculation and incubation shall be $\Delta(\lg C) \leq 1$, C is the number of viable micro-fungi of control specimen obtained from 12.1.

b) The average number of colonies of the controls immediately after inoculation shall be minimum 1×10^5 CFU and shall not be reduced more than one order of magnitude compared to the suspension of inoculation.

c) In the plate counting method, calculate the growth value (F) in accordance with Formula (2), and for yeast F shall be ≥0, for filamentous micro-fungi (dermatophyte and moulds), F shall be ≥−1.

$$F = \lg C_t - \lg C_0 \quad (2)$$

where

F is the micro-fungi growth value of the controls;

C_t is the average number of colonies of the three control specimens after incubation, expressed as CFU/ml;

C_0 is the average number of colonies of the three controls immediately after inoculation, expressed as CFU/ml.

When the conditions of a), b) and c) are satisfied, the test is judged to be effective. When the test is judged to be ineffective, a retest shall be carried out.

12.3 Calculation of antifungal activity ratio

Antifungal performance of footwear or footwear components shall be reported separately for each material or component, based on the antifungal activity ratio.

Calculate the antifungal activity ratio (R) in accordance with Formula (3), or R^* in accordance with Formula (4). Record the result in percentage with three significant figures.

$$R = \frac{C_t - T_t}{C_t} \times 100\ \% \tag{3}$$

where

C_t is the average number of colonies of three control specimens after 24 h or the specified incubation period, expressed as CFU/ml;

T_t is the average number of colonies of three test specimens after 24 h or the specified incubation period, expressed as CFU/ml.

In case when there is no control specimen available, calculate R^* by replacing C_t on Formula (3) by T_0 using Formula (4).

$$R^* = \frac{T_0 - T_t}{T_0} \times 100\ \% \tag{4}$$

where T_0 is the average number of colonies of three specimens immediately after inoculation, expressed as CFU/ml.

13 Test report

The test report shall include at least the following information:

a) reference to this document, i.e. ISO 20150:2019;

b) all information necessary for the complete identification of the treated test components, e.g. positions, areas, weights and so on;

c) the incubation conditions of the test specimens (i.e. temperature, time);

d) the test methods used for the determination of antifungal activity of different materials (i.e. dynamic or static challenge test);

e) preparation of test specimen, including pre-treatment methods, if applied, for different specimens (i.e. sterilization method);

f) the species, serial number and number of viable cells of test strains for different materials;

g) wetting agent (nonionic surfactant) and its concentration added into the test inoculums;

