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Dentistry — Evaluation of antibacterial activity of dental restorative materials, luting materials, fissure sealants and orthodontic bonding or luting materials

Médecine bucco-dentaire — Évaluation de l'activité antibactérienne des matériaux de restauration dentaire, matériaux de scellement, produits de comblement des fissures et matériaux de collage ou de scellement orthodontiques

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Contents

Page

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 document 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 106, *Dentistry,* in collaboration with the European Committee for Standardization (CEN) Technical Committee CEN/TC 55, *Dentistry,* 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.

Introduction

Due to the general applicability of *in vitro* tests for antibacterial activity and their widespread use in evaluating a large range of dental materials, it is the purpose of this document to define a scheme for testing which requires decisions to be made in a series of steps rather than to specify a single test. This should lead to the selection of the most appropriate test for a respective dental material to be evaluated.

Two categories of test are listed: extract test and direct contact test.

The choice of one or more of these categories depends upon the nature of the material to be evaluated, the potential site of use and the nature of the use of the respective material. Extract tests are primarily directed to substances leaching out from materials, whereas direct contact tests are directed to both, effects from leachable substances and surface effects. The choice of test then determines the details of the preparation of the samples to be tested, the preparation of the cultured bacteria or biofilms, and the way in which the bacteria or biofilms are exposed to the samples or their extracts.

Both categories of tests are intended to be first conducted toward planktonic cultures of bacteria and then, in case of positive results, toward bacterial biofilms.

This document proposes measurement of reduction of bacterial ability to replicate as the main method to assess antibacterial effects. Additionally, bacterial membrane damage can be assessed in order to further verify bacterial cell death and reductions in bacterial metabolic activity can be investigated as another measure of bacterial viability.

There are several means of producing results in each of these test categories. The investigator should be aware of the test categories and into which category a particular technique fits, in order to ensure the comparability with other results on similar materials both at the intra- and interlaboratory level.

Examples of quantitative test protocols for assessing reduction of bacterial ability to replicate by colony forming units (CFU) assay and for assessing bacterial membrane damage by flow cytometry and for investigating reductions in bacterial metabolic activity by MTT assay are given in this document along with guidance for the interpretation of the results.

Dentistry — Evaluation of antibacterial activity of dental restorative materials, luting materials, fissure sealants and orthodontic bonding or luting materials

1 Scope

This document specifies test methods for the evaluation of dental restorative materials, luting materials, fissure sealants and orthodontic bonding or luting materials that are claimed by their respective manufacturers to exert "antibacterial" effects.

NOTE Materials for pulp capping (e.g. calcium hydroxide formulations), endodontic filling materials, dental implants or implant systems, nightguards and additive manufactured (e.g. 3D-printed) materials are not covered in this document.

This document does not cover tests on the effectiveness of sterilization or disinfection procedures. This document cannot be used to demonstrate a lack of microbial contamination of medical devices used in dentistry.

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 1942, *Dentistry — Vocabulary*

ISO 4049, *Dentistry — Polymer-based restorative materials*

ISO 6344-3, *Coated abrasives — Determination and designation of grain size distribution — Part 3: Microgrit sizes P240 to P5000*

ISO 7405, *Dentistry — Evaluation of biocompatibility of medical devices used in dentistry*

ISO 9917-1, *Dentistry — Water-based cements — Part 1: Powder/liquid acid-base cements*

ISO 9917-2, *Dentistry — Water-based cements — Part 2: Resin-modified cements*

ISO 10993-1, *Biological evaluation of medical devices — Part 1: Evaluation and testing within a risk management process*

ISO 10993-5, *Biological evaluation of medical devices — Part 5: Tests for in vitro cytotoxicity*

ISO 10993-12, *Biological evaluation of medical devices — Part 12: Sample preparation and reference materials*

ISO 10993-18, *Biological evaluation of medical devices — Part 18: Chemical characterization of medical device materials within a risk management process*

3 Terms and definitions

For the purposes of this document, the terms and definitions given in ISO 1942, ISO 7405, ISO 10993-1 and ISO 10993-5 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
dental restorative material
material or combination of materials specially formulated and prepared for use in dentistry and/or associated procedures for restoring lost integrity of teeth or for replacing teeth

3.2
positive control material
well characterized material and/or substance that, when evaluated by a specific test method, demonstrates the suitability of the test system to yield a reproducible, appropriately positive or reactive response in the test system

[SOURCE: ISO 7405:2018, 3.3]

3.3
negative control material
well characterized material and/or substance that, when evaluated by a specific test method, demonstrates the suitability of the test system to yield a reproducible, appropriately negative, nonreactive or minimal response in the test system

Note 1 to entry: In practice, negative control materials include materials lacking the active component that is responsible for antibacterial activity or materials used in clinical practice with no antibacterial activity.

[SOURCE: ISO 7405:2018, 3.4, modified — Note 1 to entry has been replaced.]

3.4
antibacterial material
material exhibiting antibacterial activity as compared to the *negative control material* (3.3)

4 Requirements

4.1 General

The material claiming to be antibacterial shall meet one of the requirements in 4.2 and 4.3.

4.2 Extract

For tests on extract, an antibacterial material shall exhibit a median reduction of bacterial ability to replicate of at least 99,9 % (3 $\log_{10}$ steps) as compared to the negative control material when tested in accordance with 7.1.

NOTE This requirement is in line with the definitions of the American Society of Microbiology [1],[2],[3].

4.3 Direct contact

For tests by direct contact, an antibacterial material shall exhibit a median reduction of bacterial ability to replicate at least 99 % (2 $\log_{10}$ steps) as compared to the negative control material when tested in accordance with 7.2.

NOTE This requirement is in line with the definitions outlined in JIS Z 2801[4].

5 Sample preparation and control material preparation

5.1 General

The tests described in this document shall be performed on

a) an extract of the sample, and/or

b) the sample itself.

Assessment of antibacterial properties shall be made on the material prepared in accordance with the manufacturer's instructions. Before testing antibacterial properties of dental materials in accordance with this document, the physical and chemical properties of the material (and extracts) shall be assessed in accordance with ISO 10993-1 and ISO 10993-18. Before testing antibacterial properties of polymer-based restorative materials, the physical behaviour of the material should be characterized according to ISO 4049. Before testing antibacterial properties of cements, the physical behaviour of the material should be characterized according to ISO 9917-1 or ISO 9917-2, respectively.

Negative and positive control materials shall be included in each assay. If appropriate and possible, control materials should be prepared by the same procedure as the sample (see 5.2 to 5.5). In all cases, control materials shall resemble the dimensions and other material properties such as roughness of the test materials. For direct contact tests, test materials and control materials shall have a circular shape with a diameter of 10 mm and a thickness of 1 mm to be used in 48-well plates (see 7.3).

For tests on extracts, 0,2 % chlorhexidine digluconate shall be used as a positive control.[5] Additionally, to the extracts from the negative control material, nutrient broth used for bacterial culture in the respective set of experiments (see Annex A for examples) shall be used as further negative control to ensure experimental validity.

For tests by direct contact, copper plates (circular shape; diameter 10 mm; purity ≥99 %; absence of visible surface impurities) shall be used as a positive control.[6] These plates shall be ground with a P2000 paper in accordance with ISO 6344-3 in order to provide similar roughness as compared to the samples.

Negative control materials shall not exhibit any antibacterial activity. Therefore, PTFE samples shall be used that have the same size and dimensions as the test samples.

All test or control samples shall be stored in sterile water at (37 ± 1) °C after mixing/curing/milling as described by the manufacturer for 24 h prior to testing, e.g. for allowing leaching of monomers in polymers. After these initial 24 h, all test or control samples shall be tested at once and after 10 consecutive elution cycles (see 5.7.4) to provide an indication on long-term antibacterial activity.[7] If antibacterial activity is still observed after 10 elution cycles, a further test after 20 elution cycles should follow in order to demonstrate a plateau (i.e. a persisting effect) of the antibacterial activity.

Chemical analysis of the extracts should be additionally performed according to ISO 10993-18.

5.2 General requirements and recommendations for sample preparation

Sample preparation shall be in accordance with ISO 7405, ISO 10993-12, ISO 4049, ISO 9917-1 and ISO 9917-2.

For the preparation of samples, consult the respective product standards and/or the manufacturer's instructions, and follow those descriptions as closely as possible. Justify any deviation from the manufacturer's instructions. A detailed description of the sample preparation shall be included in the test report. Sample preparation shall take into account the following factors:

a) temperature;

b) humidity;

c) light exposure: samples of photosensitive materials shall be produced under the condition that ambient light does not activate them;

d) material of sample mould: ensure that the material of the sample mould and eventual lubricant used do not interfere with the setting process of the material;

e) oxygen exposure: for materials that produce an oxygen inhibition layer during hardening, both ends of the mould shall be covered with transparent oxygen barrier materials (e.g. polyester/mylar strips) during hardening;

f) samples shall be produced under aseptic conditions; in cases, where this is not possible, the samples can be sterilized by the method appropriate to the material, if necessary and possible (see 5.7.3).

5.3 Specific requirements and recommendations for light-curing materials

In accordance with ISO 7405, the following factors shall be taken into account, considering the final use of the light-curing material:

a) Material of sample mould: If possible, the material of the sample mould should be according to ISO 4049, i.e. stainless steel moulds with a white backing (white filter paper) at the bottom of the sample. In case this is not possible, reflection coefficients of materials used for sample moulds should be as close as possible to that of the oral surface to which the material is applied in order to simulate the clinical situation.

NOTE Suitable sample mould materials with reflection coefficients close to dental hard tissues can be semi-translucent or white plastic materials such as polyethylene (PE) or polytetrafluoroethylene (PTFE).

b) Light exposure: Light-curing shall be done to simulate clinical usage as closely as possible. This often requires curing from one side only but sometimes entails a two-sided cure. The cure method is material and/or process specific. In the case of one-component materials, there shall be no voids, clefts or air-bubbles present when viewed without magnification. To provide the same level of curing as would be the case in clinical usage, follow the instructions for use of the material manufacturer including the recommended powered polymerization activator, which shall include the emission wavelength region(s), the irradiance and the exposure time. This information shall be documented in the test report. Care shall be taken to ensure that the light source and operating condition conform to the instructions for use of the material manufacturer.

c) Oxygen exposure: For materials that produce an oxygen inhibition layer during light-curing, both ends of the mould shall be covered with transparent oxygen barrier materials (e.g. polyester/mylar strips) during light-curing.

d) Sample surface treatment: If the material is recommended by the manufacturer for surface finishing after curing, the sample surfaces shall be ground and polished using the recommended clinical procedures. If there are no such instructions and if required for testing, the samples shall be ground on both ends, with a P2000 paper in accordance with ISO 6344-3, after first being set against the transparent oxygen barrier material.

5.4 Specific requirements and recommendations for chemically setting materials

In accordance with ISO 7405, ISO 9917-1 and ISO 9917-2, the following factors shall be taken into account, considering the final use of the chemically setting material:

a) Mixing: Mix sufficient material to ensure that the preparation of each sample is completed from one batch. Prepare a fresh mix for each sample. The mixing shall be performed in accordance with the respective product standards, if applicable.

b) Oxygen exposure: For materials that produce an oxygen inhibition layer during chemical curing, both ends of the mould shall be covered with oxygen barrier materials (e.g. polyester/mylar strips) during curing.

c) Sample surface treatment: If the material is recommended by the manufacturer for surface finishing after curing, the sample surfaces shall be ground and polished using the recommended clinical procedures. If there are no such instructions and if required for testing, the samples shall be ground on both ends, with a P2000 paper in accordance with ISO 6344-3, after first being set against the transparent oxygen barrier material.

5.5 Specific requirements and recommendations for CAD/CAM milled or subtractive manufactured materials

The following factors shall be taken into account, considering the final use of the CAD/CAM milled or subtractive manufactured material: sample surface treatment: if the material is recommended by the manufacturer for surface finishing after CAD/CAM milling or subtractive manufacturing, the sample surfaces shall be ground and polished using the recommended clinical procedures. If there are no such instructions and if required for testing, the samples shall be ground on both ends, with a P2000 paper in accordance with ISO 6344-3.

5.6 Sterility of samples

Sterility of the samples shall be taken into account.

Samples from dental materials that are supplied sterile shall be handled aseptically throughout the test procedure.

Samples from dental materials that are normally supplied non-sterile but are sterilized before use shall be sterilized by the method recommended by the manufacturer and handled aseptically throughout the test procedure. The effect of sterilization methods or agents on the dental material should be considered in defining the preparation of the sample prior to use in the test system.

Samples from dental materials not required to be sterile in use shall be used as supplied and handled aseptically throughout the test procedure. It can be justifiable to decontaminate the test material in order to avoid cross-contamination of the bacterial culture; however, the decontamination process shall not alter the properties of the test material. An immersion in 70 % ethanol for 1 min is recommended – unless specified otherwise – to reduce cross-contamination, followed by immersion in sterile water for 1 min. Other methods for decontamination can be used if their efficacy has been proven and if it has been verified that they do not change material properties.

If non-sterile samples are used, they should be checked for bacterial cross-contamination because the contamination can lead to a false assessment of antibacterial properties.

5.7 Preparation of liquid extracts of material

5.7.1 Principles of extraction

Preparation of extracts shall be performed after a 24 h-storage in sterile water at (37 ± 1) °C following mixing/curing as described by the manufacturer and after 10 consecutive elution cycles (see 5.7.4) to provide an indication on long-term antibacterial activity[7].

If antibacterial activity is still observed for the extract after the 10th elution cycle, a further test on an extract after 20 elution cycles should follow in order to demonstrate a plateau (i.e. a persisting effect) of the antibacterial activity.

Chemical analysis of the extracts should be additionally performed after the 10^{th} and 20^{th} elution cycle according to ISO 10993-18.

Extracting conditions should attempt to simulate or exaggerate the clinical use conditions so as to determine the potential antibacterial activity without causing significant changes in the sample, such as fusion, melting or any alteration of the chemical structure, unless this is expected during clinical application. Due to the nature of certain materials (e.g. biodegradable materials), alteration of the chemical structure can occur during the extraction procedure.

NOTE The concentration of any endogenous or extraneous substances in the extract, and hence the amount exposed to the test bacteria, depends on the interfacial area, the extraction volume, pH, chemical solubility, diffusion rate, osmolarity, agitation, temperature, time and other factors.

5.7.2 Extraction vehicle

The choice of the extraction vehicle(s) taking into account the chemical characteristics of the sample shall be justified and documented. One or more of the following vehicles shall be used:

a) nutrient broth used for bacterial culture in the respective set of experiments (see Annex A for examples);

b) phosphate-buffered saline (PBS);

c) serum (for extraction of lipids).

The choice of vehicle should reflect the aim of the extraction. Nutrient broth is the preferred extraction vehicle because of its ability to extract both polar and non-polar substances.

NOTE It is important to recognize that proteins from protein-rich or serum-containing nutrient broths are known to bind, to some extent, extractables.

5.7.3 Extraction conditions

The extraction procedures shall be performed in accordance with ISO 10993-5.

The extraction shall be performed in sterile, chemically inert, closed containers by using aseptic techniques with a volume of extraction vehicle based on the exposed surface area, in accordance with ISO 10993-12.

With the exception of circumstances given below, the extraction shall be conducted under one of the following conditions and shall be applied in accordance with the material characteristics and specific conditions for use:

a) (24 ± 2) h at (37 ± 1) °C;

b) (72 ± 2) h at (37 ± 1) °C.

Other conditions which simulate the extraction that occurs during clinical use or provide an adequate measure of the antibacterial properties of the material can be used but shall be justified and documented.

Manipulation of the extract, such as by pH adjustment, filtering, centrifugation or other processing methods prior to being applied to the bacteria should be avoided because it can influence the result. If suchlike manipulation still is necessary, these details shall be recorded in the final report along with a rationale for the additional steps.

5.7.4 Consecutive elution cycles

For providing an indication on long-term antibacterial activity, consecutive elution cycles are performed by using the extraction vehicle (see 5.7.2) and extraction conditions (see 5.7.3) as described above[7].

The whole elution procedure shall be conducted by consecutively performing at least 10 single elution steps. For each single elution step, the samples shall be stored in the dark at (37 ± 1) °C for either (24 ± 2) h or (72 ± 2) h. After that period, the elution vehicle shall be renewed, and another elution

step shall be performed. This procedure shall be repeated 10 times. For this purpose, extract samples for a period of two weeks. Change the extraction vehicle four times a week in 24 h intervals and one time at 72 h. This results in 10 changes. The extracts used for testing shall be 24 h extracts and no 72 h extracts.

In the case antibacterial activity is observed for the extract after the 10th elution cycle, conduct a further 10 elution cycles and test on an extract after 20 elution cycles in order to demonstrate a plateau (i.e. a persisting effect) of the antibacterial activity. For this purpose, extract samples for a period of four weeks. Change the extraction vehicle four times a week in 24 h intervals and one time at 72 h. This results in 20 changes. The extracts used for testing should be 24 h extracts and no 72 h extracts.

According to ISO 10993-12, extracts should, if possible, be used immediately after preparation to prevent sorption on to the extraction container or other changes in composition. If an extract is stored for longer than 24 h refrigerated at 2 °C to 8 °C, then the stability and homogeneity of the extract under the storage conditions shall be verified.

5.8 Preparation of materials for direct contact tests

5.8.1 Form of samples

The preferred sample of a solid material shall have a circular shape with a diameter of 10 mm and a thickness of 1 mm to be used in 48-well plates. There should be at least one flat surface. If not, adjustments shall be made to achieve flat surfaces.

In the case of materials that have another physical state than solid (gels, etc.), they can be tested in their respective various shapes or sizes without modification in the antibacterial assays. These shapes shall be similar among all samples. In these cases, the respected volumes of nutrient broth used for bacterial culture need to be adjusted accordingly.

5.8.2 Principles of direct contact tests

Tests for direct contact shall be performed after a 24 h-storage in sterile water at (37 ± 1) °C following mixing/curing as described by the manufacturer and after 10 consecutive elution cycles (see 5.7.4) to provide an indication on long-term antibacterial activity[7].

If antibacterial activity is still observed for the material after the 10th elution cycle, a further test on the material after 20 elution cycles should follow in order to demonstrate a plateau (i.e. a persisting effect) of the antibacterial activity.

It shall be ensured that all surfaces of the samples are exposed to extraction. Samples sticking together can result in unextracted surfaces and false positive findings. Use an appropriate rack (see Figure 1) to mount samples for extraction. The volume of extraction vehicle used to extract samples shall be large, i.e. at least 10 ml/cm^2 sample surface.

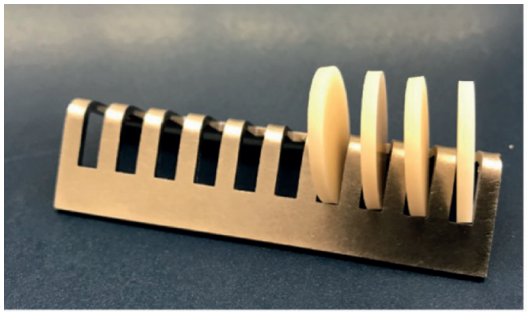

Figure 1 — Example of a rack for mounting of samples

Use one of the following extraction media:

a) nutrient broth used for bacterial culture in the respective set of experiments (see Annex A for examples);

b) PBS.

For materials that are not pH-neutral, for example, cements, products containing alkaline glasses, or that can be subject to corrosion, PBS should be used. In this instance, capacity to recharge shall be considered as well (e.g. for glass ionomer-based materials).

6 Bacterial strains, nutrient broths and preparation of bacterial cultures

Established bacterial type or reference strains are preferred and, where used, shall be obtained from recognized repositories[1]. Selection of bacterial strains shall be based on the relevance of these organisms for the area of application of the respective material (e.g. *Streptococcus* spp. for materials related to restorative dentistry or orthodontics). Due to restricted availability and potentially impaired comparability of the obtained results, no clinical bacterial isolates should be used.

Annex A summarizes recommended bacterial type or reference strains with their corresponding nutrient broths and solid growth media to be used for the set of experiments described in this document.

All experiments shall be performed in accordance with appropriate microbiological practices[2].

If a stock culture of a bacterial strain is stored, storage shall be at −80 °C or below in the corresponding nutrient broth but containing a cryoprotectant, e.g. glycerol. The nutrient broth shall meet the growth requirements of the selected bacterial type strain.

Only bacterial strains free from any cross-contamination shall be used for the test. Before use, stock cultures should be tested for the absence of cross-contamination (e.g. by MALDI-TOF analysis). Only use pure cultures. The nutrient broth used for the experiment shall be sterile and free of any cross-contamination.

Using the chosen bacterial type or reference strain and nutrient broth, prepare sufficient planktonic bacterial cultures to complete the assay. Avoid sub-culturing bacterial cultures for more than five times on agar plates or in liquid cultures because sub-culturing can change the properties of the chosen bacterial type strain.

7 Test procedures

7.1 General

Assessment of antibacterial properties shall be made on the final finished product.

A minimum of three independent experiments with at least three replicates in each experiment shall be used for samples and negative control materials and positive control materials.

1) For example, from the American Type Culture Collection (ATCC), the Deutsche Sammlung von Mikroorganismen und Zellkulturen (DSMZ) or the National Collection of Type Cultures (NCTC).

2) For example, WHO Good Practices for Pharmaceutical Microbiology Laboratories (WHO Technical Report Series, No. 961, 2011, Annex 2) [8].

7.2 Test on extracts

7.2.1 Tests on extracts toward planktonic cultures of bacteria

7.2.1.1 Principle

This test allows assessment of antibacterial properties by assessing reduction of bacterial ability to replicate in planktonic cultures and by measuring bacterial membrane damage or reduction in bacterial metabolic activity in planktonic cultures.

7.2.1.2 Apparatus, and materials and reagents

7.2.1.2.1 Apparatus

7.2.1.2.1.1 Cell culture laboratory equipment, i.e. pipettes, incubator.

7.2.1.2.1.2 96-well plates.

7.2.1.2.2 Materials and reagents

7.2.1.2.2.1 Extracts from test and control samples, see 5.6.

7.2.1.2.2.2 Nutrient broth, see Annex A.

7.2.1.2.2.3 Positive control material: 0,2 % chlorhexidine digluconate.

7.2.1.2.2.4 Negative control material, nutrient broth, see Annex A.

7.2.1.3 Procedure

Prepare overnight cultures at (37 ± 1) °C under aerobic or anaerobic conditions as appropriate for the respective culture conditions of the chosen bacterial strain (see Annex A). Adjust to a bacterial concentration that is between $2{,}5 \times 10^5$ CFU/ml and 10×10^5 CFU/ml with the same nutrient broth used to grow the bacteria. Use an optical density (measured at 600 nm) corresponding to the above bacterial concentration as a guide for the adjustment.

NOTE Although slightly varying per each bacterial strain, an optical density (measured at 600 nm) of 0,1 typically corresponds to a starting inoculum of about 10^7 CFU/ml.

Pipette a 100 µl aliquot of the adjusted overnight culture of the chosen bacterial strain into each of a sufficient number of wells of a 96-well plate for exposure to the extracts.

Pipette to each of the bacterial cultures 100 µl of

a) the original extract, and/or

b) the original extract and a dilution series of the extracts using the extract vehicle as diluent.

Alternatively, where materials of limited solubility are known or suspected to be present, dilution should be achieved by varying the original extraction ratio of sample to extraction medium.

Prepare replicate wells for both the negative control material and positive control material.

Incubate the well plates at (37 ± 1) °C under aerobic or anaerobic conditions as appropriate for the respective culture conditions of the chosen bacterial strain (see Annex A) for an appropriate period corresponding to the selected specific assay.

If biofilms form during the culture period of overnight culture with extracts, resuspend the biofilms in the supernatant (by frequently pipetting up and down followed by visual examination of the wells for confirming complete removal of the biofilms).

Determine antibacterial effects as outlined in 7.4.

7.2.2 Test on extracts toward bacterial biofilms

7.2.2.1 Principle

This test allows assessment of antibacterial properties by assessing reduction of bacterial ability to replicate in biofilms and by measuring bacterial membrane damage or reduction in bacterial metabolic activity in biofilms.

7.2.2.2 Apparatus, and materials and reagents

7.2.2.2.1 Apparatus

7.2.2.2.1.1 Cell culture laboratory equipment, i.e. pipettes, incubator.

7.2.2.2.1.2 96-well plates.

7.2.2.2.2 Materials and reagents

7.2.2.2.2.1 Extracts from test and control samples, see 5.6.

7.2.2.2.2.2 Nutrient broth, see Annex A.

7.2.2.2.2.3 Phosphate-buffered saline.

7.2.2.2.2.4 Positive control material: 0,2 % chlorhexidine digluconate.

7.2.2.2.2.5 Negative control material: Nutrient broth, see Annex A.

7.2.2.3 Procedure

Prepare overnight cultures at (37 ± 1) °C under aerobic or anaerobic conditions as appropriate for the respective culture conditions of the chosen bacterial strain (see Annex A). Adjust to a bacterial concentration that is between $2,5 \times 10^5$ CFU/ml and 10×10^5 CFU/ml with the same nutrient broth used to grow the bacteria. Use an optical density (measured at 600 nm) corresponding to the above bacterial concentration as a guide for the adjustment.

NOTE Although slightly varying per each bacterial strain, an optical density (measured at 600 nm) of 0,1 typically corresponds to a starting inoculum of about 10^7 CFU/ml.

Pipette a 200 µl aliquot of the adjusted overnight culture of the chosen bacterial strain into each of a sufficient number of wells of a 96-well plate with flat bottom for biofilm formation.

Incubate the wells at (37 ± 1) °C under aerobic or anaerobic conditions as appropriate for the respective culture conditions of the chosen bacterial strain for a total biofilm culture period of at least 48 h. Refresh the nutrient broth every 24 h by carefully discarding the old nutrient broth while not affecting the attached bacteria (mainly at the bottom of the well) and adding fresh nutrient broth.

After a total biofilm culture period of at least 48 h, carefully discard the nutrient broth and pipette to each of the bacterial biofilms 100 µl of

a) the original extract, and/or

b) the original extract and a dilution series of the extracts using the extract vehicle as diluent.

Alternatively, where materials of limited solubility are known or suspected to be present, dilution should be achieved by varying the original extraction ratio of sample to extraction medium.

Prepare replicate wells for both the negative control material and positive control material.

Incubate the well plates at (37 ± 1) °C under aerobic or anaerobic conditions as appropriate for the respective culture conditions of the chosen bacterial strain (see Annex A) for an appropriate period corresponding to the selected specific assay.

Carefully discard the nutrient broth, resuspend the biofilms in PBS (by using 200 µl PBS and frequently pipetting up and down followed by visual examination of the wells for confirming complete removal of the biofilms), transfer to new vessels and sonicate them (ultra-sonic water-bath chamber; 35 kHz; 5 min) to separate aggregated bacterial cells.

Determine antibacterial effects as outlined in 7.4.

7.3 Test by direct contact

7.3.1 Test by direct contact toward planktonic cultures of bacteria

7.3.1.1 Principle

This test allows assessment of antibacterial properties by assessing reduction of bacterial ability to replicate in planktonic cultures and by measuring bacterial membrane damage or reduction in bacterial metabolic activity in planktonic cultures.

7.3.1.2 Apparatus, and materials and reagents

7.3.1.2.1 Apparatus

7.3.1.2.1.1 Cell culture laboratory equipment, i.e. pipettes, incubator.

7.3.1.2.1.2 48-well plates.

7.3.1.2.2 Materials and reagents

7.3.1.2.2.1 Test and control samples, see 5.7.

7.3.1.2.2.2 Nutrient broth, see Annex A.

7.3.1.3 Procedure

Prepare overnight cultures at (37 ± 1) °C under aerobic or anaerobic conditions as appropriate for the respective culture conditions of the chosen bacterial strain (see Annex A). Adjust to a bacterial concentration that is between 2,5 × 10^5 CFU/ml and 10 × 10^5 CFU/ml with the same nutrient broth used to grow the bacteria. Use an optical density (measured at 600 nm) corresponding to the above bacterial concentration as a guide for the adjustment.

NOTE Although slightly varying per each bacterial strain, an optical density (measured at 600 nm) of 0,1 typically corresponds to a starting inoculum of about 10^7 CFU/ml.

Place the samples into each of a sufficient number of wells of a 48-well plate. The samples can be fixed to the bottom of the wells by using an A-silicone material used for dental impressions without any irregularities or gaps in the junction between the A-silicone material and the samples.

Carefully pipette an aliquot of the adjusted overnight culture of the chosen bacterial strain into each of the wells containing the samples until the level of the aliquot is exactly flush with the sample surface not yet covering the sample. Then add exactly 180 µl of the overnight culture per each well such that all samples are equally thick covered with the overnight culture. This results in a 2 mm thick layer of overnight culture covering the samples.

Ensure that the whole upper surface of the samples is covered with nutrient broth.

Prepare replicate wells for both the negative control material and positive control material.

Incubate the well plates at (37 ± 1) °C under aerobic or anaerobic conditions as appropriate for the respective culture conditions of the chosen bacterial strain (see Annex A) for an appropriate period corresponding to the selected specific assay.

Carefully transfer both the nutrient broth and the samples of a well into one new vessel and sonicate them (ultra-sonic water-bath chamber; 35 kHz; 5 min) to separate aggregated bacterial cells.

Determine antibacterial effects as outlined in 7.4.

7.3.2 Test by direct contact toward bacterial biofilms

7.3.2.1 Principle

This test allows assessment of antibacterial properties by assessing reduction of bacterial ability to replicate in bacterial biofilms and by measuring bacterial membrane damage or reduction in bacterial metabolic activity in bacterial biofilms.

7.3.2.2 Apparatus, and materials and reagents

7.3.2.2.1 Apparatus

7.3.2.2.1.1 Cell culture laboratory equipment, i.e. pipettes, incubator.

7.3.2.2.1.2 48-well plates.

7.3.2.2.2 Materials and reagents

7.3.2.2.2.1 Test and control samples, see 5.7.

7.3.2.2.2.2 Nutrient broth, see Annex A.

7.3.2.2.2.3 Phosphate-buffered saline.

7.3.2.3 Procedure

Prepare overnight cultures at (37 ± 1) °C under aerobic or anaerobic conditions as appropriate for the respective culture conditions of the chosen bacterial strain (see Annex A). Adjust to a bacterial concentration that is between $2{,}5 \times 10^5$ CFU/ml and 10×10^5 CFU/ml with the same nutrient broth used to grow the bacteria. Use an optical density (measured at 600 nm) corresponding to the above bacterial concentration as a guide for the adjustment.

NOTE Although slightly varying per each bacterial strain, an optical density (measured at 600 nm) of 0,1 typically corresponds to a starting inoculum of about 10^7 CFU/ml.

Place the samples into each of a sufficient number of wells of a 48-well plate. The samples can be fixed to the bottom of the wells by using an A-silicone material used for dental impressions without any irregularities or gaps in the junction between the A-silicone material and the samples.

Carefully pipette an aliquot of the adjusted overnight culture of the chosen bacterial strain into each of the wells containing the samples until the level of the aliquot is exactly flush with the sample surface not yet covering the sample. Then add exactly 180 µl of the overnight culture per each well such that all samples are equally thick covered with the overnight culture. This results in a 2 mm thick layer of overnight culture covering the samples.

Prepare replicate wells for both the negative control material and positive control material.

Incubate the wells at (37 ± 1) °C under aerobic or anaerobic conditions as appropriate for the respective culture conditions of the chosen bacterial strain (see Annex A) for a total culture period of at least 48 h. Refresh the nutrient broth every 24 h by carefully discarding the old nutrient broth while not affecting the samples containing attached bacteria. Then, add fresh nutrient broth by carefully pipetting nutrient broth into the well until the level of nutrient broth is exactly flush with the sample surface not yet covering the sample. Then add exactly 180 µl nutrient broth per each well such that all samples are equally thickly covered with the overnight culture. This results in a 2 mm thick layer of nutrient broth covering the samples.

Carefully discard the nutrient broth, remove the samples and put them into new vessels containing PBS. Then vortex (10 s) and sonicate them (ultra-sonic water-bath chamber; 35 kHz; 5 min) and repeat this procedure three times in order to separate aggregated bacterial cells (followed by visual examination of the samples for confirming complete removal of the biofilms from the samples).

Determine antibacterial effects as outlined in 7.4.

7.4 Determination of antibacterial effects

7.4.1 General

Determine the antibacterial effects by assessing reduction of bacterial ability to replicate by CFU assay (see 7.4.2).

Additionally, bacterial membrane damage can be assessed by flow cytometry (see 7.4.3) or reduction in bacterial metabolic activity can be assessed by MTT test (see 7.4.4).

7.4.2 Assessment of the reduction of the bacterial ability to replicate

7.4.2.1 Apparatus, and materials and reagents

7.4.2.1.1 Cell culture laboratory equipment, i.e. pipettes, incubator.

7.4.2.2 Materials and reagents

7.4.2.2.1 Phosphate buffered saline.

7.4.2.2.2 Solid culture media, see Annex A.

7.4.2.3 Procedure

Assess reduction of bacterial ability to replicate by means of CFU assay.

Serially dilute the bacterial suspensions from extract tests or direct contact tests tenfold in PBS (10^{-2} to 10^{-7}).

Plate aliquots from each dilution step on solid growth media (i.e. agar plates; see Annex A). Thereby, spread either 100 µl to 200 µl over an individual plate for each dilution step, or divide the plate into sextants and plate three droplets of 20 µl from each dilution step onto a sextant of the plate. Incubate the agar plates at (37 ± 1) °C under aerobic or anaerobic conditions for at least 24 h as appropriate for the respective bacterial strain. Afterwards, evaluate CFUs. Multiply the counted CFUs in each dilution step by the respective dilution factors, and calculate medians and neighbouring quartiles (25/75 % percentiles).

For tests on extracts, a median CFU-reduction of at least 99,9 % (3 $\log_{10}$ steps) as compared to the negative control material is required to use the term "antibacterial material" (as outlined in 4.2).

NOTE 1 This requirement is in line with the definitions of the American Society of Microbiology [1],[2],[3].

For tests by direct contact, a median CFU-reduction of at least 99 % (2 $\log_{10}$ steps) as compared to the negative control material is required to use the term "antibacterial material" (as outlined in 4.3).

NOTE 2 This requirement is in line with the definitions outlined in JIS Z 2801[4].

7.4.2.4 Test report

A test report shall be prepared. The test report shall include at least the following details:

a) the sample (lot number);

b) the name and address of the organization evaluating the product;

c) the site at which the evaluation took place;

d) the date of the test;

e) the standard used (i.e. ISO 3990:2023);

f) the method used;

g) the result(s) including reference to the clause which explains how these were calculated;

h) any deviations from procedure;

i) any unusual features observed;

j) the identity of the person preparing the report, including signature and date.

7.4.3 Assessment of bacterial membrane damage

7.4.3.1 Apparatus, and materials and reagents

7.4.3.1.1 Apparatus

7.4.3.1.1.1 Cell culture laboratory equipment, i.e. pipettes.

7.4.3.1.1.2 Flow cytometer, equipped for detection of fluorescence from propidium iodide and SYBR green.

7.4.3.1.2 Materials and reagents

7.4.3.1.2.1 Propidium iodide.

7.4.3.1.2.2 SYBR green.

7.4.3.1.2.3 Phosphate-buffered saline.

7.4.3.2 Procedure

Assess bacterial membrane damage by means of flow cytometry after staining with propidium iodide (PI) and SYBR green. These dyes intercalate into DNA and exhibit a strong increase in fluorescence upon nucleic acid binding. While SYBR green stains all bacteria, PI is only able to stain bacteria with damaged membranes [5],[9],[10].

NOTE 1 SYTO-9 can be used instead of SYBR Green.

Centrifuge the bacterial suspensions from extract tests or direct contact tests and resuspend them in 1 ml PBS. For Gram-positive bacteria, mix 10 µl of each sample with 984 µl PBS and 1 µl SYBR green (100 ×) and incubate for 15 min in the dark at room temperature. Subsequently, add 5 µl PI (1 µg/ml) and incubate for another 5 min. For Gram-negative bacteria, mix 10 µl of each sample with 982 µl PBS and 1 µl SYBR green (10 ×) and incubate for 15 min in the dark at room temperature. Subsequently, add 7 µl PI (1 µg/ml) and incubate for another 5 min.

After staining, immediately process the samples by a flow cytometer. Green fluorescence emitted by SYBR green can be detected on FL1, red fluorescence emitted by PI on FL3. Gate bacterial cells on FSC/SSC dot plots from which FL1/FL3 dot plots are derived. Evaluate at least 10 000 events. Bacterial membrane damage is evaluated according to the relative numbers of PI-stained or double-stained bacterial cells. Calculate medians and neighbouring quartiles (25/75 % percentiles). An increase of bacterial cells stained with PI or double-stained with PI and SYBR green as compared to the negative control material indicates a membrane-damaging effect of the respective test material.

NOTE 2 Treatment of *Streptococcus mutans* 72 h biofilms with standard antiseptics acting on bacterial membranes such as chlorhexidine digluconate 0,2 % or cetylpyridinium chloride 0,1 % for 10 min resulted in at least 70 % bacterial cells stained with PI or double-stained with PI and SYBR green[5].

7.4.3.3 Test report

A test report shall be prepared. The test report shall include at least the following details:

a) the sample (lot number);

b) the name and address of the organization evaluating the product;

c) the site at which the evaluation took place;

d) the date of the test;

e) the standard used (i.e. ISO 3990:2023);

f) the method used (including gating of the bacterial cells on FSC/SSC dot plots);

g) the result(s) including reference to the clause which explains how these were calculated;

h) any deviations from procedure;

i) any unusual features observed;

j) the identity of the person preparing the report, including signature and date.

7.4.4 Assessment of the reduction in bacterial metabolic activity

7.4.4.1 Apparatus, and materials and reagents

7.4.4.1.1 Apparatus

7.4.4.1.1.1 Cell culture laboratory equipment, i.e. pipettes.

7.4.4.1.1.2 Microplate reader, for 96-well plates, wavelength 540 nm.

7.4.4.1.2 Materials and reagents

7.4.4.1.2.1 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide.

7.4.4.1.2.2 Dimethyl sulfoxide.

7.4.4.1.2.3 Phosphate-buffered saline.

7.4.4.1.2.4 Nutrient broth, see Annex A.

7.4.4.2 Procedure

Assess reduction in bacterial metabolic activity by means of the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay.

Centrifuge (1 800 × *g*) the bacterial suspensions from extract tests or direct contact tests for 5 min, discard the supernatants and resuspend the pellet in 200 µl nutrient broth or PBS. Stain the samples by adding 22 µl of a 5 mg/ml MTT solution (dissolved in nutrient broth or PBS) to reach a final concentration of 0,5 mg/ml MTT and incubate for 1 h at 37 °C until a purple precipitate is visible. After this incubation period, centrifuge (1 800 × *g*) samples and discard the supernatants. Add 200 µl dimethyl sulfoxide (DMSO) and shake for 20 min in the dark at room temperature until the formazan crystals are completely dissolved. Transfer aliquots (180 µl) from the resulting solution and from DMSO (blanks) into wells of a 96-well plate and measure the absorbance (optical density, OD) at 540 nm using a spectrophotometer. Calculate medians and neighbouring quartiles (25/75 % percentiles) for the samples and blanks, and subtract the median value for the blank. A reduction in bacterial metabolic activity as compared to the negative control material indicates a metabolic activity-reducing effect of the respective test material.

The negative control materials should give an absorbance at 540 nm of at least 0,6 in order to be able to properly assess reductions in metabolic activity.

NOTE Culture of *Streptococcus mutans* biofilms with nutrient broth containing chlorhexidine digluconate 0,2 % for 24 h exhibited a 90 % decreased metabolic activity of the biofilms as compared to biofilms cultured without chlorhexidine digluconate 0,2 %[11].

7.4.4.3 Test report

A test report shall be prepared. The test report shall include at least the following details:

a) the sample (lot number);

b) the name and address of the organization evaluating the product;

c) the site at which the evaluation took place;

d) the date of the test;

e) the standard used (i.e. ISO 3990:2023);

f) the method used;

g) the result(s) including reference to the clause which explains how these were calculated;

h) any deviations from procedure;

i) any unusual features observed;

j) the identity of the person preparing the report, including signature and date.

8 Assessment of results

The overall assessment of the results shall be carried out by a person capable of making informed decisions based on the test data.

If a given dental material exerts antibacterial effects in the set of tests described in this document, this provides basic information on the antibacterial properties of the given material. This may not be predictive of clinical use; however, a material with no antibacterial properties as demonstrated by the set of experiments described in this document is not likely to exhibit any antibacterial properties *in vivo*.

Interpretation of the results should be based on the field and indication of clinical application of the respective material as well as on the intended duration of the antibacterial effect in the respective clinical situation. For example, an antibacterial effect that is limited to a few hours can have a worthwhile clinical impact for a dental adhesive during their respective setting periods but will not have a significant clinical impact for a resin-based composite material intended for clinical service as a direct restorative material for many years.

Testing of antibacterial activity of a given dental material shall always be seen in context with other biocompatibility aspects. Therefore, testing for antibacterial activity of a given dental material should always be accompanied by cytotoxicity testing of this respective material according to ISO 7405 and ISO 10993-5 as well as chemical analysis of this respective material according to ISO 10993-18.

9 Final test report

At the conclusion of testing, a final test report shall be compiled, presenting the test procedures listed in this document. This final test report shall include at least the following items of information:

a) name of the product;

b) name of the manufacturer;

c) place of manufacture (if available);

d) lot number tested;

e) the use by date of this product (if one is given);

f) the ISO Standard with its publication date (i.e. ISO 3990:2023);

g) the name of the organization evaluating the product and the site at which this took place;

h) the date of the evaluation;

i) bacterial strain(s), justification of the choice and source(s);

j) name of the company and batch of the nutrient broth used;

k) assay method and rationale;

l) consecutive elution cycles procedure;

m) extraction procedure (if appropriate) and, if possible, the nature and concentration of the leached substance(s);

n) negative, positive and other control materials;

o) observed (or not observed) antibacterial activity and other observations including appropriate statistical analysis;

p) any other relevant data necessary for the assessment of the results;

q) the identity of the person preparing the report, including signature and date.