INTERNATIONAL STANDARD

ISO 19090

First edition
2018-01

Tissue-engineered medical products — Bioactive ceramics — Method to measure cell migration in porous materials

Produits médicaux issus de l'ingénierie tissulaire — Céramiques bioactives — Méthode de mesure de la migration cellulaire dans les matériaux poreux

Reference number
ISO 19090:2018(E)

ISO copyright office
CP 401 • Ch. de Blandonnet 8
CH-1214 Vernier, Geneva, Switzerland
Tel. +41 22 749 01 11
Fax +41 22 749 09 47
copyright@iso.org
www.iso.org

Published in Switzerland

Contents

Page

Foreword

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

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

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 on 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 the following URL: www.iso.org/iso/foreword.html.

ISO 19090 was prepared by Technical Committee ISO/TC 150, *Implants for surgery*, Subcommittee SC 7, *Tissue-engineered medical products*.

Introduction

"Bioactive ceramics" are widely used in orthopaedic and dental fields due to their bioactivities and bioaffinities. Porous bioactive ceramics are designed as bone void fillers, and cell migration from tissue into their pores is an expectation for effective repair of bone defects; thus, they are one of the promising candidates for cell scaffolds for bone tissue engineering medical products.

To clarify the clinical safety and usefulness of these bioactive ceramics, physical, chemical and biological properties must be examined. In the methods used, animal tests are the ultimate and essential methods to examine biological properties of bioactive ceramics; however, numbers of both animals and animal tests must be reduced under the concept of 3R (Replacement, Reduction and Refinement)[3].

The first and most important property for porous biomaterials including bioactive ceramics is cell migration capability, because cell proliferation, differentiation, tissue formation and tissue maturation in and surroundings of porous biomaterials do not occur without cell migration.

Currently, two different cell-seeding methods are used for estimating "cell migration" property: One is dropping a cell suspension on the top surface of a porous material. This method tests the penetration ability of the "cell suspension" under gravity and estimates the number of cells that migrate into and are held within the porous material. The other method is shaking a porous material in the cell suspension. This method also tests the penetration ability of the "cell suspension" like the above method but uses shaking to drive the cells into the porous scaffolds. Both methods test the abilities of cell penetration and retention only, and do not test the intrinsic ability of the cell to migrate simulating what happens *in vivo*. Body fluid itself can sufficiently carry cells across a minor gap between the implanted material and the host bone. Accordingly, no cell migration test methods have been reported that mimic cell behaviour *in vivo*.

When porous bioceramics are implanted into bone defect, cells migrate into the pore to form new bone. In this process, migration of osteoblasts mainly plays important roles for osteoconduction. That is to say, no osteoconduction nor bone formation can occur without osteoblast migration.

Therefore, it is imperative to establish a quantifiable method to measure cell migration potential of porous bioactive ceramics in a manner similar to how cells behave *in vivo*, in order to evaluate their potential appropriately as materials for tissue-engineered medical products.

Tissue-engineered medical products — Bioactive ceramics — Method to measure cell migration in porous materials

1 Scope

The document specifies the test method to be followed for measuring and documenting the cell migration ability of porous bioactive ceramic materials.

This document is not applicable to porous materials that have low or no cell adhesion properties, for instance synthetic polymers and metals. These types of materials will require longer times to allow effective transfer and migration of cells from the cultured substrate to the test specimen. To minimize influences of cell passages, cell kinds, differences in cell culture consumables including culture medium and fetal bovine serum etc., the method uses a porous bioactive ceramics, which is clinically and widely used in each country, as a reference material for calculation of relative migration distance.

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

3 Terms and definitions

For the purposes of this document, the terms and definitions given in ISO 10993-5 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 https://www.electropedia.org/

3.1
bioceramics
ceramics used that enhance biological functions when implanted in the human body

3.2
bioactive ceramics
bioceramics (3.1) with direct bone bonding property when implanted into bone defect

3.3
biomaterial
material used in or to be used in medical and dental field

3.4
full confluent
cell cultured dish is almost completely (≈95 % to 100 %) covered with a monolayer of cells

3.5
complete medium
cell culture media that is recommended for the chosen cell type by the supplier of the cells with all required supplements cell culture medium that is confirmed by the user that the cells used in the test proliferate well without any mutation

3.6
osteoblast-like cell
established cell lines which widely recognised to have “osteogenic activity”

3.7
cell group
group of cells composed of at least five cells where the distance between each cell is less than the cell width

4 Principle

The cell migration property of porous biomaterials has been estimated by measuring cell numbers that are seeded in the porous body by two methods, however, they measure penetration of cell suspension and cell attachment, not migration by cells themselves.

The present method is very simple and effective to measure cell migration by themselves. Cells confined in a confluent layer of a culture dish are able to further migrate and proliferate from this layer into the porous bioceramic materials that are placed on top of the culture dish as shown in Figure 1. To minimize influences of cell passages, cell types, differences in cell culture consumables including culture medium and serum, etc., the method uses a reference material that porous bioactive ceramics commercialised and used clinically with good clinical results in each country for calculation of relative migration distance.

a) Before: No cells exist in a test material **b) After: Cells move up to a test material through pores**

Key

1 culture dish filled with cell culture medium

2 cells adhered on a bottom of culture dish

3 test material

Figure 1 — Schematic drawing of test method

After the initial transfer of cells onto the material interface, they start to migrate into pores of the materials. This migration distance will differ in relation to the materials properties and chemical stability, surface morphologies, and pore structures similar to what is seen *in vivo* and which mimics the initial part of the bone regeneration process.

Giemsa staining is a very stable and easy method to stain cells for this method.

Linear longest migration distance of a valid cell measured from the cross section of the Giemsa stained porous bioactive ceramics is well reflected cell migration *in vivo*.

5 Test specimen

5.1 Shape and dimensions

The shape of the specimen should be a disk (10 ± 0,2) mm in diameter and (2 ± 0,1) mm in thickness.

5.2 Number of test specimens

At least five pieces are required for the comparison between two (reference and test) materials.

5.3 Reference material

Commercially available porous bioactive ceramics in each country that are confirmed as good bone void filler by basic researches and/or clinical results. The reference material available in multiple countries is recommended.

6 Procedures

6.1 Measurement of thickness and diameter of specimen

The thickness and diameter of specimen shall be measured with a calliper or a micrometer.

6.2 Sterilization of test specimens

Specimens shall be sterilized by a method which does not affect the material quality before the test.

6.3 Deaeration of test specimens

Specimens shall be immersed in 5 mL complete medium in a centrifuge tube which can be sealed tightly with a lid. After an 18 to 21 gauge needle connected with a 20 mL syringe is inserted through the lid of the tube, the specimens shall be deaerated by pulling the plunger of the syringe back completely with tapping on the tube for 2 min to 3 min to eliminate bubbles.

6.4 Cell culture

Osteoblast-like cells shall be seeded on 6-well cell culture plates or ⌀35 mm cell culture dishes and cultured to full confluency. Seeding cell number will be decided by the preliminary test to obtain confluency within 3 days. MG-63 and MC3T3-E1 are recommended for osteoblast-like cells. To obtain confluent cells within 3 days in 6-well culture plate, seeding of $6,0 \times 10^4$ per well for MG63 or $8,0 \times 10^4$ per well for MC3T3-E1 will be needed. Cells shall be observed under a phase-contrast microscope for 2 days to 3 days after seeding to check cell confluency and to avoid cell overconfluency.

NOTE The number of cells is counted by conventional methods using the hemocytometer, described in ISO 13366-1 or cell counting devices, described in ASTM F2149–01.

The number of cells to be seeded to reach full confluency within 3 days should be confirmed by the preliminary test, if the cell line other than recommended in this document will be used in this test.

When wells (dishes) are covered with confluent cells, 3 wells (dishes) shall be stained with Giemsa to record the cell confluent status before migration test as follows: After being fixed with 2 % glutaraldehyde overnight, Giemsa solution with appropriated concentration shall be prepared with PBS, and 2,5 mL of the solution shall be added to each well (dish) and incubated for 3 min at room temperature. Then the staining solution shall be removed and washed with 2,5 mL distilled water for 3 times. Stereoscopic micrograph of each well or dish shall be taken at x10 or maximum available magnification so that the bottom of plates or dishes can be observed in the same scope.

6.5 Placing specimens on the cell layer

When cells have reached full confluency, one deaerated specimen shall be placed on the cell layer and the SUS316 stainless steel double ring with 10 mm in ring outer diameter and 0,8 mm to 1,0 mm in wire diameter, shown in Figure 2, shall be put on the specimen to brace in place. Both shall be placed with forceps without dropping nor agitation of the medium. There can be some roughness and unevenness on the surface of the specimen due to manufacturing process. Choose a smooth surface of the specimen to place on the cell layer.

When transferring the plates (or dishes) into the CO_2 incubator, take care not to move the specimen inadvertently since it could impair the close contact of the specimen with the cell layer. The cells shall be cultured another 3 days without medium change or any other procedure.

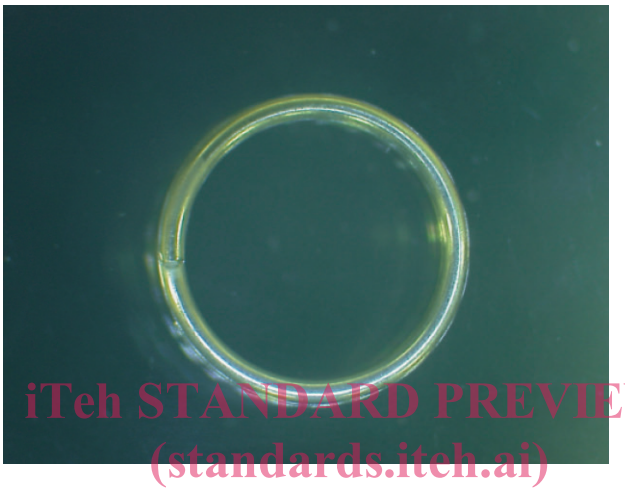


Figure 2 — Stainless steel double ring

Set the weight on the sample to ensure close contact between the sample and the confluent cell layer. However, if the influence of the extra weight on the cell is unclear, the acute cytotoxicity should be evaluated by cell proliferation assay as follows, to ensure that there is no effect.

a) wash the weight with ultrapure water with applying ultrasonic for 1 h followed by rinsing with ultrapure water 3 times.

b) dry heat sterilize the weight at 160 °C for 3 h.

c) place the weight on the full confluent cell layer as same as the cell layer to be used in the test and observed cell shape and numbers surroundings of the weight with phase-contrast microscope daily for at least 3 days.

6.6 Treatments after cell culture

The specimen shall be harvested 3 days after incubation. To prevent detachment of cells from the specimen, place the bottom side (cell contact surface) of the specimen up after harvesting. The specimen shall be washed with PBS for 3 times and fixed with 2 % glutaraldehyde overnight. After fixation, the specimen shall be washed with PBS for 3 times and immersed in 10 mL of Giemsa solution with appropriate concentration at room temperature. After 3 min, the sample shall be transferred to another vessel and washed with 10 mL of distilled water. The wash is repeated 3 times.

If this staining method does not work or is very different from the manufacturer's instruction, follow the staining protocol in the manufacturer's instruction.

Wells or dishes after harvesting specimen shall be washed with PBS for 3 times and fixed with 2 % glutaraldehyde overnight. After fixation, the wells or dishes shall be washed with PBS for 3 times and pour 2,5 mL of Giemsa solution with appropriate concentration at room temperature. After 3 min, the solution will be removed and washed with distilled water. The wash is repeated 3 times. Then,

the staining solution shall be removed and washed with 2,5 mL of distilled water for 3 times. Cell migrations from the edge of the cell transferred areas on the well or dish are observed, if the specimen and stainless steel double ring are not affected cell viability. Further, if cells in the specimen placed area showed no change in shape and numbers but did not transfer well, low "flatness" of the specimen can be the reason.

6.7 Positive and negative controls

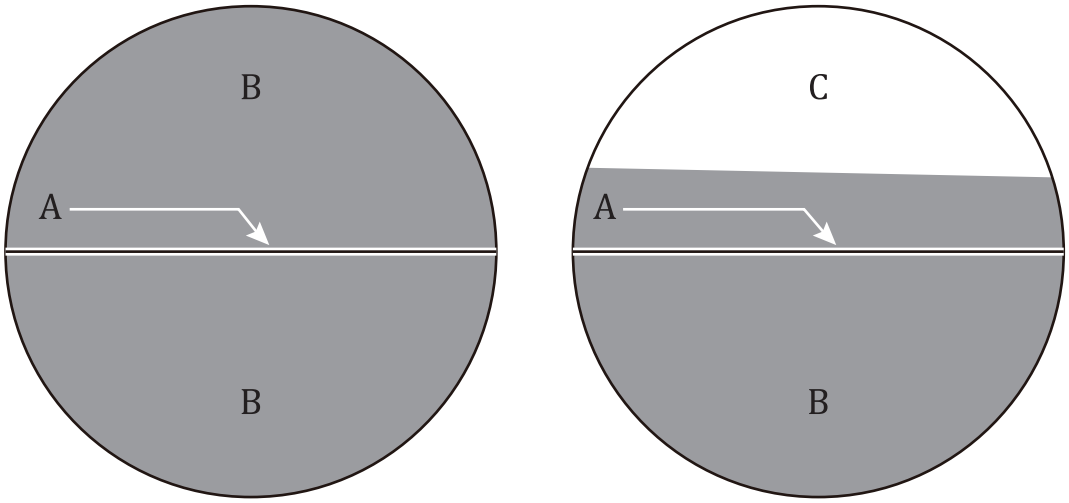
Including positive and negative controls for the assay signal can be useful for each assay. For example, an appropriate negative control can be to conduct the measurement in the absence of cells. The scaffolds can be placed in dishes without cells, incubated with culture medium, washed, stained, imaged and scored. As an example of an appropriate positive control, the scaffolds can be directly seeded with cells using a pipette, incubated in medium to let the cells adhere (possibly 4 h or 24 h), washed, stained, imaged and scored.

These controls provide assurance that the assay is working effectively and help with interpreting the results. In the case where cells migrate into the test scaffolds, it is important to demonstrate that the negative controls did not score positively for cell migration. This provides evidence that the background staining of the scaffold and the scoring procedure, among other things, are reliable. In the case where there is poor migration into the test scaffolds, it is important to demonstrate that the positive controls scored positively for cells. This provides evidence that cells were viable, the stain was effective, and the scoring procedure was reliable (among other things). Including positive and negative controls in the assay makes it possible to interpret the results and improves confidence in the conclusions.

6.8 Observations of cells that migrated into a specimen

Annexes A and B show typical results and are useful to understand practical results and procedures as referred in the following procedures.

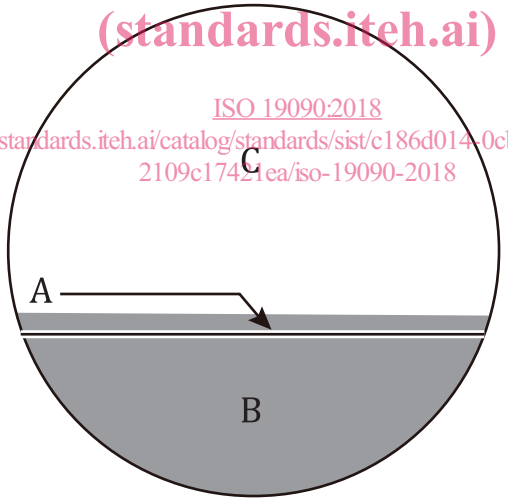
The cell-contact (bottom) side of the specimen shall be observed with a stereoscopic microscope. Stereoscopic microphotographs of the bottom side shall be taken. The specimen shall be cut into two pieces using a thin scalpel commonly used for eye surgery. Depending on the flatness of bottom surface of the specimen, stained area might have irregular distribution and shape as shown in Figures A.5 and B.3; therefore, the cutting position shall be determined from the stained specimen interface (bottom) that had been in direct contact with the cell layer; and the incision shall be made along the longest determined length through the darkest stained area of the specimen (Figures 3 to 6).



Key

A cutting position
B stained area
C non-stained area

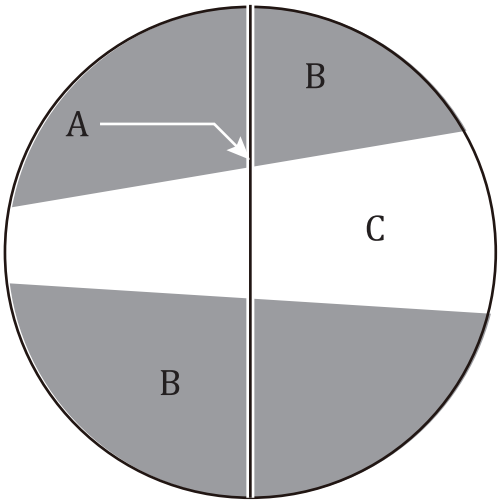
Figure 3 — Schematic drawing to determine cutting line for specimen surface of which stained area is more than a half



Key

A cutting position
B stained area
C non-stained area

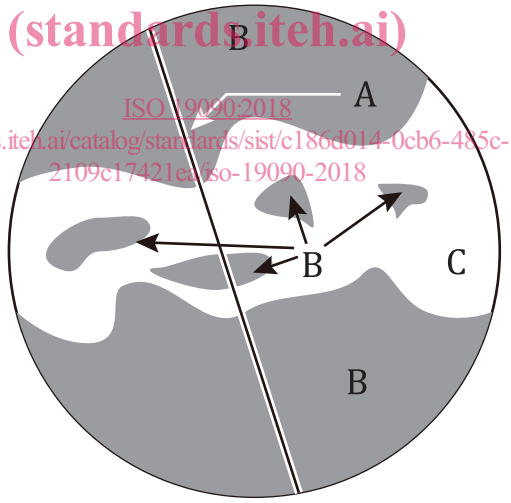
Figure 4 — Schematic drawing to determine cutting line for specimen surface of which stained area is less than a half



Key

A cutting position
B stained area
C non-stained area

Figure 5 — Schematic drawing to determine cutting line for specimen surface of which stained areas are partitioned



Key

A cutting position
B stained area
C non-stained area

Figure 6 — Schematic drawing to determine cutting line for specimen surface of which stained areas are distributed

The cross section should be observed preferably with a stereoscopic microscope. Stereoscopic microphotographs of the cross section of the specimen and micrometre at the same magnification shall be taken.

NOTE 1 Specimens are cleaved with a scalpel that is normally used for eye surgery. If it is impossible to cleave specimens with a scalpel, the specimen is cut with a diamond cutter or similar apparatus using phosphate buffered saline as lubricant.