INTERNATIONAL STANDARD

**ISO 16604**

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# Clothing for protection against contact with blood and body fluids — Determination of resistance of protective clothing materials to penetration by blood-borne pathogens — Test method using Phi-X174 bacteriophage

*Vêtements de protection contre les contacts avec le sang et les fluides corporels — Détermination de la résistance à la pénétration par des pathogènes véhiculés par le sang des matériaux entrant dans la fabrication des vêtements de protection — Méthode d'essai utilisant le bactériophage Phi-X174*



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## Foreword

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

International Standards are drafted in accordance with the rules given in the ISO/IEC Directives, Part 2.

The main task of technical committees is to prepare International Standards. Draft International Standards adopted by the technical committees are circulated to the member bodies for voting. Publication as an International Standard requires approval by at least 75 % of the member bodies casting a vote.

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.

ISO 16604 was prepared by Technical Committee ISO/TC 94, *Personal safety — Protective clothing and equipment*, Subcommittee SC 13, *Protective clothing*. It is based on ASTM F1671-97b.

# Introduction

Workers, primarily those in the health care profession, involved in treating and caring for individuals injured or sick, can be exposed to biological liquids capable of transmitting disease. These diseases, which may be caused by a variety of microorganisms, can pose significant risks to life and health. This is especially true of blood-borne viruses which cause hepatitis [hepatitis B virus (HBV) and hepatitis C virus (HCV)] and acquired immune deficiency syndrome (AIDS) [human immunodeficiency viruses (HIV)]. Since engineering controls cannot eliminate all possible exposures, attention is placed on reducing the potential of direct skin contact through the use of protective clothing.

This International Standard is concerned with protective clothing and related protective devices designed to protect against the penetration of blood or body fluids.

Given the variety of health care settings, activities, and the potential for exposure to blood or body fluids, the barrier requirements for protective clothing materials will change with the application.

This International Standard describes a hydrostatic pressure test for measuring the viral penetration resistance of clothing materials to a surrogate virus. The choice of an appropriate test method depends on the specific application of protective clothing and its intended use. A risk assessment should be performed to determine the level of risk for determining the appropriate test method.[1]

This test method does not apply to all forms or conditions of blood-borne pathogen exposure. Users of this test method should review modes for worker/clothing exposure and assess the appropriateness of this test method for their specific applications. This test method has been specifically defined for modelling the viral penetration of hepatitis (B and C) and human immunodeficiency viruses transmitted in blood and other potentially infectious body fluids. The surrogate microbe, Phi-X174 bacteriophage, used in this test method, is similar to HCV in size and shape but also serves as a surrogate for HBV and HIV. Inferences for protection from other pathogens should be assessed on a case-by-case basis.

This test method addresses only the performance of materials or certain material constructions (e.g. seams) used in protective clothing. This test method does not address the design, overall construction and components, or interfaces of garments or other factors which may affect the overall protection offered by the protective clothing. It is emphasized that the test does not necessarily simulate conditions that clothing materials are likely to be exposed to in practice. The use of test data should therefore be restricted to broad comparative assessment of such material according to their viral penetration resistance characteristics.

Testing prior to degradation by physical, chemical, and thermal stresses which could negatively impact the performance of the protective barrier, could lead to a false sense of security. Consider tests which assess the impact of sterilization, storage conditions, and shelf life on the penetration resistance for disposable products, and the effects of laundering and sterilization on the penetration resistance for reusable products. The integrity of the protective barrier can also be compromised during use by such effects as flexing and abrasion.[1] It is also possible that pre-wetting by contaminating materials such as alcohol and perspiration also compromises the integrity of the protective barrier. If these conditions are of concern, evaluate the performance of protective clothing materials for Phi-X174 bacteriophage penetration following an appropriate preconditioning technique representative of the expected conditions of use.

Medical protective clothing materials are intended to be a barrier to blood, body fluids, and other potentially infectious materials. Many factors can affect the wetting and penetration characteristics of body fluids, such as surface tension, viscosity, and polarity of the fluid, as well as the structure and relative hydrophilicity or hydrophobicity of the materials. The surface tension range for blood and body fluids (excluding saliva) is approximately 0,042 N/m to 0,060 N/m.[2] In order to help simulate the wetting characteristics of blood and body fluids, the surface tension of the Phi-X174 bacteriophage challenge suspension is adjusted to approximate the lower end of this surface tension range. The resulting surface tension of the Phi-X174 bacteriophage challenge suspension is (0,042 ± 0,002) N/m.

Part of this method for exposing the protective clothing material specimens with Phi-X174 bacteriophage challenge suspension involves pressurization of the test cell to 14,0 kPa (in Procedures A and B). This hydrostatic pressure has been documented to produce test results that correlate with visual penetration results that are obtained with a human factors validation.[3] Some studies, however, suggest that mechanical pressures exceeding 345 kPa can occur during clinical use.[4] [5] Therefore, it is important to understand that this test method does not simulate all the physical stresses and pressures that are exerted on protective clothing garments during actual use. Procedures C and D use a stepped pressurization approach with pressures up to 20,0 kPa. These procedures simulate a range of possible pressures for ranking material performance.

# Clothing for protection against contact with blood and body fluids — Determination of resistance of protective clothing materials to penetration by blood-borne pathogens — Test method using Phi-X174 bacteriophage



## 1 Scope

This International Standard describes a laboratory test method for measuring the resistance of materials used in protective clothing to penetration by blood-borne pathogens. This test method uses a surrogate microbe under conditions of continuous liquid contact. Protective clothing "pass/fail" determinations are based on the detection of viral penetration at a specific hydrostatic pressure using the ISO 13994 test apparatus.

This test method is not always effective in testing protective clothing materials having thick, inner liners which readily absorb the challenge fluid.

This test method involves a sensitive assay procedure. Because of the length of time required to complete this test method, it might not be suitable for use as a material or protective clothing quality control or assurance procedure.

## 2 Normative references

The following referenced documents are indispensable for the application 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 304, *Surface active agents — Determination of surface tension by drawing up liquid films*

ISO 3696:1987, *Water for analytical laboratory use — Specification and test methods*

ISO 3801, *Textiles — Woven fabrics — Determination of mass per unit length and mass per unit area*

ISO 5084, *Textiles — Determination of thickness of textiles and textile products*

ISO 13994, *Clothing for protection against liquid chemicals — Determination of the resistance of protective clothing materials to penetration by liquids under pressure*

ISO 16603, *Clothing for protection against contact with blood and body fluids — Determination of the resistance of protective clothing materials to penetration by blood and body fluids — Test method using synthetic blood*

## 3 Terms and definitions

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

**3.1**
**agar**
semi-solid culture medium used to support the growth of bacteria and other microorganisms

**3.2**
**assay**
analysis of a mixture to determine the presence of or concentration of a particular component

NOTE In this test method, the component being analysed is a microorganism, Phi-X174 bacteriophage.

**3.3**
**assay fluid**
sterile liquid used to wash the test material surface to determine microbiological penetration

NOTE In this test method, the assay fluid is nutrient broth and the bacterial virus is the Phi-X174 bacteriophage. The assay fluid is used to wash the Phi-X174 bacteriophage from the normal inside material surface of the test specimen.

**3.4**
**bacteriophage**
type of virus which infects bacteria

NOTE In this test method, the bacteriophage is Phi-X174. The Phi-X174 bacteriophage is not pathogenic to humans, but serves to simulate viruses that are pathogenic to humans.

**3.5**
**blood-borne pathogen**
infectious secreted or excreted bacterium, virus, or other disease-inducing microbe carried in blood or other body fluids

NOTE For the purpose of this International Standard, the primary blood-borne pathogens include hepatitis B virus (HBV), hepatitis C virus (HCV), and human immunodeficiency virus (HIV). Other microorganisms should be considered on a case-by-case basis.

**3.6**
**body fluid**
any liquid produced (secreted or excreted) by the body

NOTE For the purpose of this International Standard, body fluids include those liquids potentially infected with blood-borne pathogens, including, but are not limited to, blood, semen, vaginal secretions, cerebrospinal fluid, synovial fluid, peritoneal fluid, amniotic fluid, saliva in dental procedures, and any body fluid that is visibly contaminated with blood, and all body fluids in situation where it is difficult or impossible to differentiate between body fluids.

**3.7**
**body fluid simulant**
liquid which is used to act as a model for human body liquids

NOTE In this test method, the body fluid simulant is bacteriophage nutrient broth, which is intended as a model for human body liquids as its approximates the lower end of the surface tension range for blood and body fluids (excluding saliva), (0,042 ± 0,002) N/m.

**3.8**
**challenge suspension**
liquid containing an agent that is used to test the penetration resistance of materials

NOTE In this test method, the challenge suspension is the bacteriophage challenge suspension; a nutrient broth containing the Phi-X174 bacteriophage.

**3.9**
**lawn**
〈microbiology〉 cloudy, uniform growth of bacteria in a thin layer of top agar in a petri dish

NOTE In this test method, *Escherichia coli C. (E. coli C)* has been selected as the bacterium used to produce the lawn.

**3.10**
**lysis**
disintegration or destruction of whole bacterial cells

NOTE In this test method, the lysis of the host bacteria, *E. coli C.,* is caused by Phi-X174 bacteriophage.

**3.11**
**medium**
**media**
nutrient system for the cultivation of cells or organisms

NOTE In this test method, the term media is used to describe mixtures compounded to support the growth of specific microorganisms, for example, bacteriophage nutrient broth and top agar.

**3.12**
**morphology**
form and structure of a particular organism

**3.13**
**nutrient broth**
liquid medium

NOTE In this test method, the nutrient broth is the bacteriophage nutrient broth which is used to culture the host bacteria, *E. coli C.*, and to aid in manipulating the Phi-X174 bacteriophage through the various stages of the procedure, such as suspending the Phi-X174 bacteriophage for challenging the test material in the penetration cell, assaying the normal inside test material surface, and if required, making dilutions of assay fluid for plating.

**3.14**
**penetration**
flow of a liquid through closures, porous materials, seams and holes or other imperfections in a protective clothing material on a non-molecular level

**3.15**
**plaque**
⟨virology⟩ visible, clear area which is theoretically the result of the infection and lysis of host cells by a single viable virus

NOTE In this test method, the term plaque is used to describe a visible, clear area, in the lawn of *E. Coli C.* in top agar which is theoretically the result of a single viable Phi-X174, where the bacteria have been destroyed by bacteriophage infection and lysis.

**3.16**
**plaque-forming unit**
**PFU**
virus particle capable of producing plaques by infecting and lysing bacteria in a lawn in top agar

**3.17**
**plate**
⟨microbiology⟩ Petri dish containing culture medium

**3.18**
**protective clothing**
item of clothing that is specifically designed and constructed for the intended purpose of isolating all or part of the body from a potential hazard; or, isolating the external environment from contamination by the wearer of the clothing.

**3.19**
**surrogate microbe**
microorganism which is used to act as a simulant for other microorganisms which are pathogenic to humans

NOTE In this test method, the surrogate microbe is the Phi-X174 bacteriophage, intended as a model for HCV and to simulate both HBV and HIV.

**3.20**
**titre**
quantity of a substance required to react with, or to correspond to, a given amount of another substance

NOTE In this test method, titre is used to describe the concentration of viable bacteriophage as measured in plaque-forming units per millilitre (PFU/ml).

**3.21**
**virus**
minute infectious agent, which lacks independent metabolism and is only able to replicate within a living host cell

**3.22**
**viral penetration**
penetration of a material by a virus

NOTE In this test method, the physical translocation of Phi-X174 bacteriophage through closures, seams, pores, and pinholes or other imperfections in materials used in protective clothing.

**3.23**
**viral resistant**
referring to materials which impede viral penetration under specified laboratory test conditions and detection methods

NOTE In this test method, protective clothing materials which demonstrate "pass" results are considered to be resistant to viral penetration.

## 4 Principle

A specimen is subjected to a nutrient broth containing a virus in a test apparatus as specified in ISO 13994 for a specified time and pressure sequence. Visual detection of penetration is supplemented with an assay procedure that will detect viable viruses which penetrate the material even when liquid penetration is not visible. Any evidence of viral penetration for a test specimen constitutes failure.

This test method requires a working knowledge of basic microbiological techniques.

## 5 Microorganisms and reagents

**5.1 Bacteriophage Phi-X174 (ATCC 13706-B1)**, used at a challenge titre of at least $1{,}0 \times 10^8$ PFU/ml (plaque-forming units per millilitre).

NOTE The surrogate microbe, Phi-X174 bacteriophage, was selected as the most appropriate model for blood-borne pathogens because of its small size, spherical (icosahedral) morphology, environmental stability, non-human infectivity, high assay sensitivity, rapid assay, and high titre. The Phi-X174 bacteriophage has no envelope and is one of the smallest known viruses (0,027 µm in diameter).

**5.2** ***Bacteria E. coli*** (ATCC 13706).

**5.3 Purified water**, grade 3 in accordance with ISO 3696:1987.

**5.4 Nutrient broth**.

**5.5 Calcium chloride ($CaCl_2$)**.

**5.6 Potassium chloride (KCl)**.

**5.7 Sodium hydroxide (NaOH)**, 2,5 mol/l solution.

**5.8 Surfactant**, Polysorbate 80.

**5.9 Bacto-agar**.

## 6 Apparatus and materials

**6.1 Penetration test cell**, as specified in ISO 13994, to restrain the specimen during contact with the pressurized challenge fluid.

In the test cell, the specimen acts as a partition separating Phi-X174 bacteriophage challenge suspension from the view side of the test cell. It consists of a cell body that is fastened to a cell support. The cell body has a capacity of approximately 60 ml for the Phi-X174 bacteriophage challenge suspension. A flange cover, with an open area to allow visual observation, and a transparent cover are included. The cell body has a top port for filling and a drain valve for draining the penetration test cell. Other items, such as a fitting to allow attachment of the air line to the top port in the cell body, gaskets, and the retaining screen are also required. A diagram of the penetration test cell and apparatus are provided in Figures 1 and 2.

### 6.2 Other equipment

**6.2.1 Thickness gauge**, suitable for measuring thickness to the nearest 0,02 mm.

**6.2.2 Retaining screen**, comprising a smooth finish plastic or metal square mesh screen to support extensible or elastomeric materials, meeting the following specifications:

a) open area of $> 50$ %,

b) deflection of the test specimen is limited to $\leqslant 5{,}0$ mm.

**6.2.3 Air pressure source**, capable of providing air at $\left(20{,}0\,^{+2}_{0}\right)$ kPa.

**6.2.4 Incubator**, capable of sustaining a temperature range of (36 ± 1) °C.

**6.2.5 Water bath**, capable of achieving a temperature range of (45 ± 2) °C.

**6.2.6 Balance**, with a precision of 0,001 g.

**6.2.7 Vortex mixer**.

**6.2.8 Refrigerator**, capable of maintaining a temperature range of (5 ± 3) °C.

**6.2.9 Autoclave**, capable of maintaining (122 ± 1) °C and (214 ± 7) kPa absolute.

**6.2.10 Stopwatch**, or electronic timer.

**6.2.11 Orbital shaker**.

**6.2.12 pH meter**, sensitive to 0,1 pH units.

**6.2.13 Inoculating loop**.

**6.2.14 Torque wrench**, capable of applying a torque of 13,6 N·m.

**6.2.15 Spectrophotometer**, capable of measuring absorbed light at 640 nm.

**6.2.16 Centrifuge**, capable of an acceleration of 10 000 $g$.

### 6.3 Laboratory glassware

**6.3.1 Petri dishes**, sterile, 15 mm × 100 mm.

**6.3.2 Pipettes**, sterile, of 1 ml, 5 ml, 10 ml capacity.

**6.3.3 Test tubes**, 13 mm × 100 mm.

**6.3.4 Test tube rack**.

**6.3.5 Glass bottles**, sterile, with a capacity of 100 ml to 500 ml.

**6.3.6 Micropipettes**, capable of delivering 2 µl accurately and consistently.

# 7 Test specimens

## 7.1 Selection

**7.1.1** Select specimens from single material samples or individual protective clothing items (following sterilization, if applicable) consisting of either a single layer or a composite of multiple layers that is representative of an actual protective clothing construction with all layers arranged in proper order.

If in the design of an item of protective clothing, different materials or thicknesses of material are specified at different locations, select specimens from each location.

If in the design of an item of protective clothing, seams are claimed to offer the same protection as the base materials, test additional specimens containing such seams.

Cut each material specimen into squares with a minimum dimension of 70 mm. A 75 mm square is preferred.

Test three specimens taken at random from each material, composite, area (in the case of heterogeneous design), or other condition.

If this procedure is used for quality control or to support broad product claims concerning the viral-resistant properties of materials used in protective clothing, proper statistical design and analysis of larger data sets than those specified in this test method should be performed. Examples of acceptable sampling plans are found in references such as ISO 2859-1[8].

**7.1.2** It is possible that protective clothing materials incorporating an impervious layer between two fabric layers are sensitive to false positive failures by wicking at the edges. Seal the edges of the test specimens to prevent “wicking” modes of failure using an adhesive, parafilm, paraffin wax, or adhesive-backed foam prior to testing. Seal only the edges of the test specimens, leaving the centre 57 mm area open for testing. Do not allow sealants to intrude, block, or occlude the structure of the test specimen in the test area, as this may compromise the test procedure. Choose sealants and sealing methods that are compatible with the protective clothing materials.

## 7.2 Preparation of test specimens

Condition each protective clothing specimen for a minimum of 24 h by exposure to a temperature of (21 ± 5) °C and a relative humidity of (60 ± 10) %.

If warranted, use other preconditioning options, such as sterilization, to assess possible degradation mechanisms of protective clothing.



# 8 Procedure

## 8.1 Preparation of test media

### 8.1.1 Bacteriophage nutrient broth (Phi-X)

Prepare bacteriophage nutrient broth using the following:

- Bacto-tryptone (8,0 ± 0,1) g
- Potassium chloride (5,0 ± 0,06) g
- Calcium chloride (0,2 ± 0,003) g
- Purified water (5.3) (1 000 ± 12,5) ml

— Surfactant (5.8) (0,1 ± 0,001 25) ml

Adjust pH of the bacteriophage nutrient broth to (7,3 ± 1) using 2,5 mol/l sodium hydroxide.

Dilute 1 volume of 0,1 % surfactant with 9 volumes of bacteriophage nutrient broth. To ensure adequate mixing, prior to sterilization, heat the bacteriophage nutrient broth while stirring in the surfactant. A final concentration of 0,01 % surfactant is recommended to adjust the surface tension to (0,042 ± 0,002) N/m.

Sterilize the bacteriophage nutrient broth in the autoclave.

Measure the resulting surface tension of the sterile solution in accordance with ISO 304. Do not use bacteriophage nutrient broth unless the corrected surface tension is within the (0,042 ± 0,002) N/m range.

#### 8.1.2 Bottom agar (Phi-X)

Prepare the bottom agar using the following:

— Bacto-agar (15,0 ± 0,19) g

— Nutrient broth (8,0 ± 0,1) g

— Potassium chloride (5,0 ± 0,06) g

— Purified water (5.3) (1 000 ± 12,5) ml

— Calcium chloride (1,0 ± 0,012 5) ml (to be added after autoclaving the bottom agar)

Prepare sterile calcium chloride by autoclaving a 1 mol/l solution of calcium chloride in purified water.

Adjust pH of the bottom agar to (7,3 ± 1) using 2,5 mol/l sodium hydroxide.

Sterilize the bottom agar in the autoclave.

#### 8.1.3 Top agar (Phi-X)

Prepare the top agar using the following:

— Bacto-agar (7,0 ± 0,09) g

— Nutrient broth (8,0 ± 0,1) g

— Potassium chloride (5,0 ± 0,06) g

— Purified water (5.3) (1 000 ± 12,5) ml

— Calcium chloride (1,0 ± 0,012 5) ml (to be added after autoclaving the bottom agar)

Prepare sterile calcium chloride by autoclaving a 1 mol/l solution of calcium chloride in purified water.

Adjust pH of the top agar to (7,3 ± 1) using 2,5 mol/l sodium hydroxide.

Sterilize the top agar in the autoclave.

### 8.2 Preparation of controls

Use the following controls concurrently with the testing of each protective garment or protective garment material.

a) Aerosol/airborne contamination controls: settle plates or other appropriate means to determine background aerosol/airborne counts for the Phi-X174 bacteriophage.

Use the following controls at least one time per day:

b) Negative test sample control: samples made of a heavy gauge monolithic film such as a medical packaging polyester film.

c) Positive test sample control: samples made of a microfiltration medium with a pore size slightly larger, (0,050 ± 0,005) µm, than the mean diameter of the Phi-X174 bacteriophage, 0,027 µm.

### 8.3 Determination of material compatibility

Conduct compatibility testing of protective clothing materials which are suspected of affecting the titre of the bacteriophage challenge suspension.

a) Test three specimens representing each material to be tested.

b) With the penetration cell placed horizontally on the lab bench, aseptically insert the sterile specimen in the penetration cell with the normal outside surface toward the cell reservoir.

c) Assemble the sterile components of the cell as shown in Figure 1.

d) Torque the bolts in the penetration test cell to 13,6 N·m each.

e) With the penetration cell remaining in the horizontal position on the lab bench, place a 2,0 µl aliquot of the Phi-X174 bacteriophage in the bacteriophage nutrient broth, containing a total of 900 PFU to 1 200 PFU, near the middle of each specimen.

f) Prepare a control by adding a 2,0 µl aliquot of the Phi-X174 bacteriophage suspension directly to 5,0 ml of sterile bacteriophage nutrient broth.

g) After 10 min, quantitatively assay as described in 8.9.

h) Calculate the ratio of the control assay titre to the test material assay titre.

i) Fix the titres of the Phi-X174 bacteriophage challenge suspension prepared (see 8.4), which is also used for the test exposure procedure (see 8.8), as being equal to the ratio calculated in h) multiplied by (2 to 3) × $10^8$ PFU/ml. If the calculated ratio is above 5,0, the maximum titre for the bacteriophage challenge suspension shall be 1 × $10^9$ PFU/ml.

### 8.4 Procedure for preparation of bacteriophage suspension

Prepare the bacteriophage suspension using the following steps:

a) Using an inoculating loop, inoculate 10 ml to 25 ml of bacteriophage nutrient broth in a 250 ml conical flask with *E. coli C.* Incubate the bacterial culture overnight at (36 ± 1) °C with shaking at (225 ± 25) r/min.

b) Prepare a 1:100 dilution of the overnight bacterial culture in 100 ml of fresh bacteriophage nutrient broth in a 1 l conical flask. Incubate the flask at (36 ± 1) °C with shaking at (225 ± 25) r/min. Grow bacterial culture to a density of (3 ± 1) × $10^8$ CFU/ml (about 3 h). This cell density corresponds to a 0,3 to 0,5 absorbance reading (at 640 nm) as measured on a spectrophotometer.

c) Inoculate the bacterial culture with 5 ml to 10 ml of the Phi-X174 bacteriophage stock having a titre of 1,0 × $10^9$ PFU/ml to 1,0 × $10^{10}$ PFU/ml. Ensure that the ratio of bacteriophage to bacteria cells is between 0,1 and 2,0.

d) Incubate the inoculated bacterial culture at (36 ± 1) °C with vigorous shaking for 1 h to 5 h or until lysis is complete. Lysis is considered complete when the absorbance reading at 640 nm stops decreasing.

e) Centrifuge the culture for 20 min at 10 000 *g* to remove large cell debris. Decant the supernatant into a clean tube.

f) Filter bacteriophage-containing supernatant suspension through a 0,22 µm filter to purify the bacteriophage solution.

g) Determine the titre of the bacteriophage stock and store at (5 ± 3) °C. The bacteriophage titre obtained is typically in the range of (5,0 ± 2) × $10^{10}$ PFU/ml.

h) Prepare the bacteriophage challenge suspension by diluting the phage stock in the bacteriophage nutrient broth to the concentration required by 8.3. Verify the final concentration of the phage using the assay procedure (as specified in 8.9).

### 8.5 Preparation of settle plates

If elected, place settle pates in strategic locations during the aseptic test specimen insertion, filling, testing, draining, and assay operations, to help identify potential problems associated with aerosolized or airborne Phi-X174 bacteriophage. Prepare settle plates as follows.

a) Dispense 2,5 ml of sterile molten top agar into sterile test tubes and hold the temperature of the top agar at (45 ± 2) °C. Prepare one test tube for each settle plate.

b) Add 100 µl of an overnight culture of *E. coli C.* to each top agar tube.

c) Vortex tubes well and pour contents over the surface of the bottom agar plates.

d) Allow agar to solidify. Settle plates may be used immediately.

e) After use, incubate settle plates with the assay plates for the test sample replicates and the positive and the negative controls.

### 8.6 Preliminary material measures

Measure the thickness of each specimen prior to sterilization (if sterilized) to the nearest 0,02 mm in accordance with ISO 5084.

Measure the mass of each specimen prior to sterilization (if sterilized) and report the mass per unit area to the nearest 10 g/$m^2$ in accordance with ISO 3801.

Use a retaining screen when support of extensible or elastomeric materials is required.

Determine the compatibility of each test material as specified in 8.3.

### 8.7 Preparation of test cell

Autoclave the penetration test cell at (122 ± 1) °C and (214 ± 7) kPa absolute for 15 min. Allow the penetration test cell to cool to room temperature.

With the cell placed horizontally on the lab bench, aseptically insert the specimen in the penetration cell with the normal outside surface toward the cell reservoir which will be filled with the Phi-X174 bacteriophage challenge suspension.

Assemble the sterile components of the penetration test cell as follows.

a) Place gaskets between the penetration cell and the test specimen, the specimen and the retaining screen (if used); and the retaining screen and the flange cover as shown in Figure 1.

b) Close the penetration cell by putting on the flange cover and transparent cover (a sterile, clear plastic film may be used in lieu of the transparent cover). A compressible polytetrafluoroethylene gasket material is recommended for use between the cell body and the test specimen to help prevent leakage.

Torque the bolts in the penetration test cell to 13,6 N·m each.

Mount the penetration cell in test apparatus in a vertical position as shown in Figure 2 (drain valve down), but do not connect the air line to the cell.

Close the drain valve.

### 8.8 Exposure of material to bacteriophage challenge suspension

Expose each material specimen to the bacteriophage challenge suspension using the following steps.

a) Select and follow an appropriate procedure from Table 1.

b) Carefully fill the chamber of the penetration cell through the top port with approximately 60 ml of Phi-X174 bacteriophage challenge suspension (a sterile syringe or funnel is useful). If liquid appears to penetrate through the test specimen at anytime during the test, go straight to step i).

c) Connect the air line to the penetration cell.

d) Observe the viewing surface of the specimen at the end of each specified pressure and time interval for the appearance of liquid penetration or other evidence of wetness. If elected, record the time of visible liquid penetration.

e) If there is no visible penetration, continue on to the next step of the time and pressure protocol.

f) The pressure in the challenge side of the test cell shall be changed to the next level at a rate of (3,5 ± 0,5) kPa/s.

g) Hold the pressure constant at each specified level for the specified time.

h) When returning to atmospheric pressure, turn off the pressure and open the cell valve to the vent position.

i) At the end of the time period, open the drain valve and drain the penetration cell of bacteriophage challenge suspension.

j) Dilute and assay the Phi-X174 bacteriophage challenge suspension collected from at least the last penetration cell of each set of replicates after the test to be sure that there has been no loss of bacteriophage virulence during the test.

k) With the cell placed horizontally on the lab bench, remove the transparent cover.

l) Immediately after removing the cover, slowly add 5,0 ml sterile nutrient broth with 0,01 % surfactant, onto the exposed surface of the specimen's normal inside surface. Gently swirl or rock the penetration cell for approximately 1 min to ensure contact of this assay fluid with the entire viewing surface of the test specimen. Remove the assay fluid, as soon as possible with a sterile pipette to a sterile vial. Some materials absorb the assay fluid, requiring a larger volume wash. If a larger volume is necessary, be sure to adjust the calculation of PFU/ml in the test report.

m) Assay immediately as specified in 8.9. A longer period may lapse between collection of the assay fluid and the actual assay if stability of the bacteriophage in the assay fluid can be demonstrated.

n) Disassemble the apparatus and clean the penetration cell. Disinfect the air lines periodically to prevent contamination. Clean the penetration cell by rinsing with a 10 % bleach solution, and subsequently autoclaving it at (122 ± 1) °C and (214 ± 7) kPa absolute for 15 min.

o) Test the remaining specimens.

**Table 1 — Time and pressure protocols**

| Procedure | Pressure and time sequence | Remarks |
|---|---|---|
| **A** | 0 kPa for 5 min,<br>followed by 14 kPa for 1 min,<br>followed by 0 kPa for 4 min.<br><br>A retaining screen is not used to support the sample. | Used for selecting critical zone materials and components to limit exposure in situations involving presence of a large amount of blood or body fluids, a direct liquid contact, pressing and leaning. |
| **B** | 0 kPa for 5 min,<br>followed by 14 kPa for 1 min,<br>followed by 0 kPa for 4 min.<br><br>A retaining screen is used to support the sample. | Procedure B involves the use of a retaining screen to support extensible or elastomeric materials. When distortion of the test material is suspected of causing failure with Procedure A, Procedure B may be used. |
| **C** | 0 kPa for 5 min,<br>followed by one of the following:<br>1,75 kPa for 5 min; or<br>3,5 kPa for 5 min; or<br>7 kPa for 5 min; or<br>14 kPa for 5 min; or<br>20 kPa for 5 min.<br><br>A retaining screen is not used to support the sample. | Used for selecting critical zone materials and components of protective apparel, to limit exposure in situations involving the presence of blood or body fluid, and different levels of contact pressures.<br><br>NOTE A selection as to the level of protection required is made, based on a task analysis and the degree of exposure anticipated. |
| **D** | 0 kPa for 5 min,<br>followed by one of the following:<br>1,75 kPa for 5 min; or<br>3,5 kPa for 5 min; or<br>7 kPa for 5 min; or<br>14 kPa for 5 min; or<br>20 kPa for 5 min.<br><br>A retaining screen is used to support the sample. | Procedure D involves the use of a retaining screen to support extensible or elastomeric materials. When distortion of the test material is suspected of causing failure with Procedure C, Procedure D may be used. |
| When using Procedure C or D, the visual endpoint found in ISO 16603 can be used to determine the appropriate time and pressure sequence. The highest pressure with no visible penetration in ISO 16603 should be used for this International Standard. | | |



## 8.9 Procedure for quantification of assay fluid

Quantify the number of bacteriophage in the assay fluid using the following procedure:

a) Dispense 2,5 ml of sterile molten top agar into sterile test tubes and hold top agar at (45 ± 2) °C.

b) Prepare duplicate plates for each assay collected for each replicate and control.

c) Prepare inoculate tubes by adding 0,5 ml of the assay medium fluid from each specimen to a top agar tube immediately after removing the tube from the heat.

d) Add 100 µl of an overnight culture of *E. coli C* to each of the inoculate tubes.

e) Vortex tubes well and pour over the surface of the bottom agar plates.

f) Allow agar to solidify and incubate at (36 ± 1) °C until plaques are clearly visible, usually for at least 6 h.

g) Observe for the presence of plaques and interpret result as described in 8.10.

h) If quantification is needed, and the total number of plaques is too great to count, prepare a series of 1:10 dilutions in bacteriophage nutrient broth of the assay fluid and assay for bacteriophages as in steps a) to g).

### 8.10 Interpretation of tests results

Interpret the results and use control results as follows:

a) Consider the test invalid if any background counts (above zero) are observed when settle plates are used.

NOTE The test may be valid if the test specimens do not show penetration.

b) Consider the test valid, when negative test sample controls pass the test with no detectable transfer (< 1 PFU/ml) of the Phi-X174 bacteriophage.

c) Consider the test valid when positive test control samples fail the test.

d) Material specimens that exhibit no detectable transfer (< 1 PFU/ml) of the Phi-X174 bacteriophage in the assay titre pass the test.

When qualifying the integrity of materials, supporting broad product claims, or using the test as a quality control and assurance procedure, the test should be modified for larger data sets with proper statistical design and analysis.

False positives can be minimized by following standard microbiological regimes and proper aseptic techniques. If test results are in doubt, repeat the test using statistically valid sample methods.

## 9 Test report

The test report shall include the following information:

a) a reference to this International Standard (ISO 16604:2004);

b) the manufacturer's identity and the identification of the material, lot number and date of receipt;

c) a description of the sampling method used, for example if the material was taken from roll goods or garments;

d) the characteristics of each material:

   1) the composition of the material types of fibres and coatings,

   2) for materials taken from garments, indicate the following:

      i) composite,

      ii) type of seam,

      iii) other conditions tested,

      iv) the position on the garment of each specimen;

e) the thickness of each material specimen and the average thickness of the material tested (in millimetres);

f) the mass per unit area of each material specimen and the average mass per unit area of the material tested (in grams per square metre);

g) the procedure used (from Table 1);

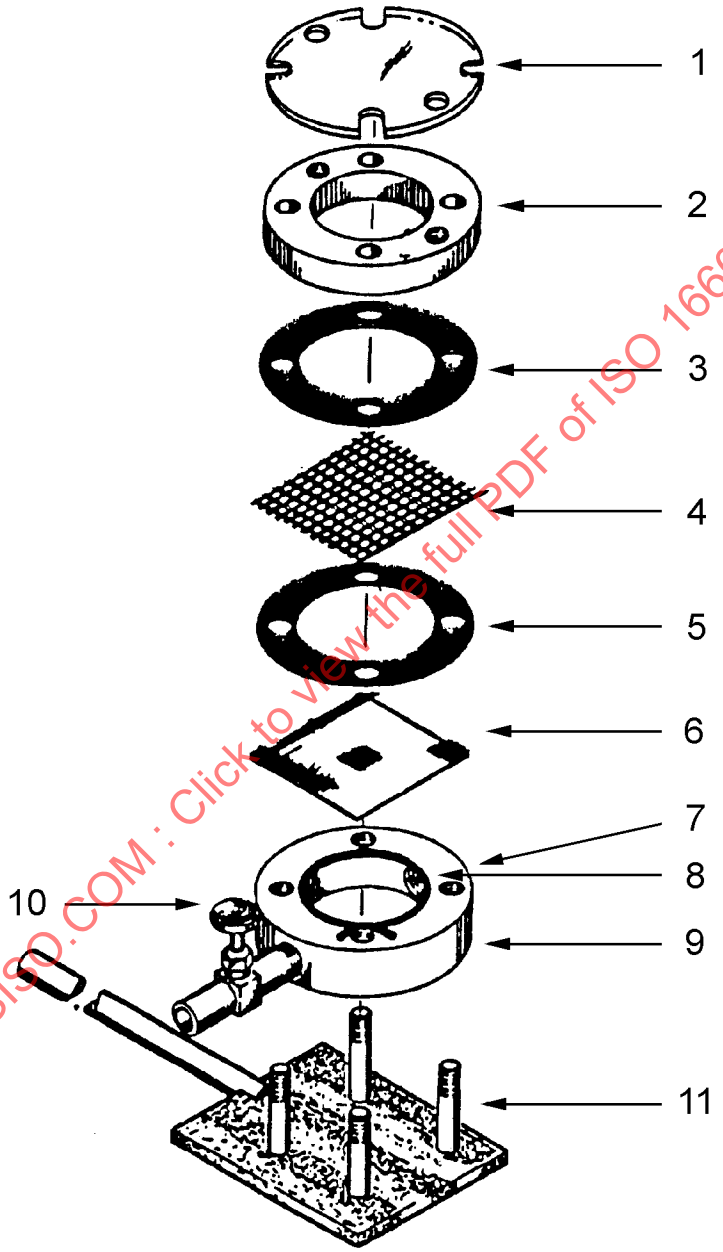
h) the type and specification for the support screen, if used;

i) the ratio calculated during compatibility testing (see 8.3);

j) the results of all controls to assure test validity, including the number of PFU for each settle plate by location, and the number of PFU/ml of assay fluid for each negative control and positive control, and the starting and ending bacteriophage challenge titre;

k) for each specimen, the “pass” or “fail” result and for each negative and positive control, if the test was terminated due to visible liquid penetration;

l) the time of visible liquid penetration for each specimen (if elected to report).






**Key**

1 transparent cover
2 flange cover
3 gasket (specimen exposure Procedures B and D)
4 retaining screen (specimen exposure Procedures B and D)
5 gasket
6 test sample
7 top port
8 expanded PTFE gasket material
9 cell body
10 drain valve
11 cell support

**Figure 1 — Penetration test cell with retaining screen** (exploded view)