भारतीय मानक **IS 18468 (Part 5) : 2024**

Indian Standard

(*Superseding IS/ISO/TS 15883-5 : 2005*)

धोनेवाले-कीटाणुशोधक

भाग 5 सफाई प्रभावकारिता को प्रदर्शित करने के लिए प्रदर्शन अपेक्षाएँ और परीक्षण पद्धति मानदंड

(ISO 15883-5 : 2021, संशोधित)

Washer-Disinfectors

Part 5 Performance Requirements and Test Method Criteria for Demonstrating Cleaning Efficacy

(ISO 15883-5 : 2021, MOD)

ICS 11.080.10

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Hospital Equipment and Surgical Disposal Sectional Committee, MHD 12

NATIONAL FOREWORD

This Indian Standard (Part 5) which is Modified adoption to ISO 15883-5 : 2021 'Washer-disinfectors — Part 5: Performance requirements and test method criteria for demonstrating cleaning efficacy' issued by the International Organization for Standardization (ISO) was adopted by Bureau of Indian Standards on the recommendation of the Hospital Equipment and Surgical Disposal Sectional Committee and after approval of the Medical Equipment and Hospital Planning Division Council.

This Indian Standard supersedes IS/ISO/TS 15883-5 : 2005 which was published in 2019 as an Identical adoption of ISO/TS 15883-5 : 2005. After publication of this standard, IS/ISO/TS 15883-5 : 2005 stands withdrawn.

The text of ISO standard, has been approved as suitable for publication as an Indian Standard without deviations. Certain conventions are however not identical to those used in Indian Standards. Attention is particularly drawn to the following:

a) Wherever the words 'International Standard' appear referring to this standard, they should be read as 'Indian Standard'; and
b) Comma (,) has been used as a decimal marker while in Indian Standards, the current practice is to use a point (.) as the decimal marker.

In this adopted standard, reference appears to certain International Standards for which Indian Standards also exist. The corresponding Indian Standards which are to be substituted in their respective places are listed below along with their degree of equivalence for the editions indicated:

International Standard	*Corresponding Indian Standard*	*Degree of Equivalence*
ISO 10993-1 Biological evaluation of medical devices — Part 1: Evaluation and testing within a risk management process	IS 17932 (Part 1) : 2023 Biological evaluation of medical devices: Part 1 Evaluation and testing within a risk management process (ISO 10993-1 : 2018, MOD)	Modified
ISO 10993-5 Biological evaluation of medical devices — Part 5: Tests for in vitro cytotoxicity	IS/ISO 10993-5 : 2009 Biological Evaluation of Medical Devices: Part 5 Tests for *in-vitro* cytotoxicity	Identical
ISO 15883-1 Washer-disinfectors — Part 1: General requirements, terms and definitions and tests	IS/ISO 15883-1 : 2006 Washer disinfectors: Part 1 General requirements, terms and definitions and tests	Identical

The standard also makes a reference to the BIS Certification Marking of the product, details of which are given in National Annex F.

For the purpose of deciding whether a particular requirement of this standard is complied with the final value, observed or calculated, expressing the result of a test or analysis shall be rounded off in accordance with IS 2 : 2022 'Rules for rounding off numerical values (*second revision*). The number of significant places retained in the rounded off value should be same as that of the specified value in this standard.

Contents

Page

Introduction

Testing of cleaning efficacy is a key aspect of establishing satisfactory performance of a washer-disinfector (WD). This testing includes type testing under simulated use conditions. In addition to type testing, performance qualification testing is performed under clinical use conditions.

The cleaning efficacy of washer-disinfectors has historically been demonstrated by referring to different test soils and methods that have been used in several different countries. This document gives requirements for standardized methods for demonstration of cleaning efficacy, including examples of test soils. The individual requirements for the various types of washer-disinfectors and processing procedures can vary, but this document provides the basis for the demonstration of cleaning efficacy.

Cleaning efficacy testing is performed in the WD and with associated accessories in two phases:

— type testing, under simulated use conditions, with defined test soils and their analytes, soiling methods and test surfaces/medical devices/product representative of design and intended applications;

— performance qualification testing under clinical conditions with load(s) that are soiled with the most challenging soil from clinical use.

This document excludes the verification of cleaning of product that could have been exposed to prions, the causative agent in transmissible spongiform encephalopathies such as Creutzfeldt-Jakob disease (CJD).

Indian Standard

WASHER-DISINFECTORS

PART 5 PERFORMANCE REQUIREMENTS AND TEST METHOD CRITERIA FOR DEMONSTRATING CLEANING EFFICACY

(ISO 15883-5 : 2021, MOD)

1 Scope

This document specifies procedures and test methods used to demonstrate the cleaning efficacy of washer-disinfectors (WD) and their accessories intended to be used for cleaning of reusable medical devices.

NOTE 1 The requirements can be used for washer-disinfectors intended for use with other articles used in the context of medical, dental, laboratory, pharmaceutical and veterinary practice.

NOTE 2 This document does not apply to the activities to be performed by the manufacturers of reusable medical devices.

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 10993-1, *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 15883-1:—[1)], *Washer-disinfectors — Part 1: General requirements, terms and definitions and tests*

3 Terms and definitions

For the purposes of this document, the terms and definitions given in ISO 15883-1 and the following apply.

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

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

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

3.1
action level
value from monitoring that necessitates immediate intervention

[SOURCE: ISO 11139:2018, 3.5]

1) Under preparation. Stage at the time of publication: ISO/DIS 15883-1:2020.

3.2
alert level
value from monitoring providing early warning of deviation from specified conditions

[SOURCE: ISO 11139:2018, 3.11]

3.3
analyte
chemical substance that is the subject of chemical analysis

[SOURCE: ISO 11139:2018, 3.12]

3.4
clean
visually free of soil and below specified levels of *analytes* (3.3)

[SOURCE: ISO 11139:2018, 3.45]

3.5
clinical use
use of a health care product during a procedure on a patient

Note 1 to entry: This encompasses all steps prior to processing in a WD.

[SOURCE: ISO 11139:2018, 3.49, modified – Note 1 to entry has been added]

3.6
load
product, equipment, or materials to be processed together within an operating cycle

[SOURCE: ISO 11139:2018, 3.155]

3.7
product
tangible result of a process

EXAMPLE Raw material(s), intermediates(s), sub-assembly(ies), health care product(s)

[SOURCE: ISO 11139:2018, 3.217]

3.8
rinsing
removing process residues through displacement by, and dilution with, water

[SOURCE: ISO 11139:2018, 3.237]

3.9
simulated use
use that mimics the intended use of the medical device

3.10
soil
natural or artificial contamination on a device or surface following its use or simulated use

[SOURCE: ISO 11139:2018, 3.257]

3.11
surrogate product
item designed to represent product in process simulations and which is comparable with the actual product

[SOURCE: ISO 11139:2018, 3.291]

3.12
test soil
formulation designed for use as a substitute for a contaminant or debris found on a device after use

[SOURCE: ISO 11139:2018, 3.300]

3.13
washing
removal of contaminants from surfaces by means of an aqueous fluid

[SOURCE: ISO 11139:2018, 3.321]

4 Performance requirements

4.1 General

4.1.1 In addition to the requirements below (see 4.1.3 to 4.1.5), the relevant cleaning performance requirements of the subsequent parts of ISO 15883 that apply to the washer-disinfector type shall apply.

4.1.2 In addition to the tests specified in 4.1.4 and 4.1.5), the relevant cleaning tests of the subsequent parts of ISO 15883 that apply to the washer-disinfector type shall apply.

NOTE See for example ISO 15883-7[5].

4.1.3 The process conditions for cleaning, e.g. stages, temperatures, pressure, flow, process chemicals, quality and quantity of water, used to confirm conformance of the WD with the requirements of this standard shall be defined in accordance with ISO 15883-1:—, 4.1.12 and 8.2 b).

NOTE Refer to ISO 15883-1:—, 5.23 and ISO 15883-4 [4] for water quality.

4.1.4 Tests of cleaning efficacy shall be performed on the defined cleaning stages, including, where appropriate, flushing, rinsing, etc. (see 5.2). Cleaning stages shall be specified according to ISO 15883-1:—, 4.1. It shall be verified and documented that the full cleaning stage does not interfere with analyte detection. During tests of cleaning efficacy, the WD shall be operated without any disinfection or drying stage and should not affect the efficacy and safety of the WD process.

4.1.5 Cleaning efficacy testing shall be performed in the WD and with accessories specified for the particular load in two phases:

a) type testing under simulated use conditions with defined test soil(s), including the analyte(s) and representative test load(s) (see 4.4.1),

b) performance qualification testing with worst-case load(s) soiled by clinical use (see 4.4.1), or if justified (5.4.2), with surrogate product.

4.2 Test soil considerations

4.2.1 The rationale for the choice of test soil(s) and soiling method(s) shall be justified and documented. Test soil formulations may be chosen or developed based on a review of the literature and demonstration of its relevance based on the use of the medical device/product in clinical practice (see Annex A and the Bibliography).

NOTE The test soils for the load, chamber walls and load carriers can be different.

4.2.2 The protein-based test soil shall conform to the performance criteria specified in B.2.

NOTE Sample result sheets for data entry are provided in Annex E.

4.2.3 The choice of test soil, its method of application, and conditioning (e.g. drying) shall simulate worst-case clinical use conditions of the load.

a) Composition of the test soil shall include the analyte(s) representative of soiling likely to be encountered during intended use of the product at a quantity justified by 4.2.1, and if applicable, any associated procedural material(s) used on the product during its clinical use, that are intended to be cleaned (e.g. contrast media, lubricants, etc.).

b) The method of test soil application shall simulate the conditions of use of the product, for example, cauterization or heating that present a greater challenge to cleaning, and/or pressure gradients that could facilitate the penetration of material into various parts of the product. Parts of the product identified as the most difficult to clean shall be soiled (see 4.3).

c) After application of the test soil on the product or test pieces, consideration shall be given to transport and dwell time conditions (e.g. temperature, time, humidity) for the product from point of use to place of processing, and if applicable, any pre-treatment (see 5.1.2.2).

d) The composition of the soil shall be characterized and the most difficult soil elements (e.g. lipids, adhesives, insoluble proteins, etc.) shall be identified and considered in the validation strategy to ensure that the validation activities demonstrate effective removal of the soil.

4.2.4 The method of test soil extraction, recovery efficiency, and detection of analytes shall be validated and specified. Validation of the recovery shall demonstrate the ability to reduce analyte below the action level.

An appropriate percent recovery is greater than 70 %, unless otherwise justified (see 5.1.3.2).

4.3 Load considerations

4.3.1 Load(s), including their respective product that represent typical and worst-case, clinical use conditions, shall be defined and justified. Such load(s) shall be used for cleaning efficacy and process residuals for type testing and performance qualification tests [see also ISO 15883-1:—, 8.1 b) and ISO 15883-4[4]. The load(s) shall be considered appropriate for the type of washer-disinfector being tested.

NOTE For type testing and performance qualification testing, when justified, the load can be surrogate product, which could be used for tests if they are shown to be representative of the prescribed load.

4.3.2 Consideration shall be given to any applicable physical characteristics of the product type(s) and patient contact area, including but not restricted to:

— lumens;

— valves;

— crevices;

— hinges and joints;

— rough and irregular surfaces;

— material composition, including porosity;

— junctions and dead ends;

— internal moveable parts (e.g. cables).

These design characteristics are at a greater risk of accumulation and retention of soil and shall be specifically considered in the estimation and risk assessment of cleaning efficacy and cleaning endpoints of the entire product.

NOTE See ASTM F3357[18] for additional detail on medical device design and cleaning.

4.3.3 Any necessary pre-treatment of the product specified in its IFU, e.g. manual pre-cleaning or disassembly, shall be included as part of the test procedure.

4.4 Cleaning efficacy test criteria

4.4.1 General

Cleaning efficacy shall be determined by visual examination (see 4.4.2) and by the quantitative detection of protein (see 4.4.3.2, Note and Annex B).

For invasive medical devices, at least one other validated quantitative analytical test method shall be used to measure another analyte(s) in addition to protein for type testing.

Non-invasive medical devices shall require visual examination only.

NOTE 1 Some non-invasive medical devices can represent higher levels of risk e.g. infant formula bottles, contact tonometers.

NOTE 2 A validated qualitative method (see References [22],[52],[54],[70] and[88]) can be used for routine testing when the detection level of this method is below the alert level assay criteria given in 4.4.3.

NOTE 3 Typical analytes are given in 4.4.3.2 and 4.4.3.3 (also see Bibliography).

NOTE 4 Refer to ISO 14971[3] for approach to risk assessment to support justification.

4.4.2 Visual examination

Visual examination shall demonstrate the absence of visible soil on all observable surfaces of the load(s), following cleaning stage(s). This requirement does not apply where adequate visual inspection of the surfaces of the product is not possible due to its configuration.

NOTE Adequate visual inspection requirements can include:

— defined instructions for inspection;

— adequate illumination;

— inspection aids, if applicable (e.g. lighted magnification boroscope);

— viewing distance.

Refer to EN 13018[10] and ASTM E3106[16] for additional information on visual inspection.

4.4.3 Assay criteria

4.4.3.1 General

Acceptance criteria for analytes are specified in terms of both an alert level and an action level.

The alert and action levels for protein and other analytes are specified in 4.4.3.2 and 4.4.3.3. Their action levels are the maximum criteria for acceptable cleaning efficacy during testing, but the target values are given as the alert levels. When both alert and action levels are specified, if analytes are detected at values between the two levels, they shall be investigated, but considered to pass cleaning requirements.

For the purpose of conforming to the requirements of 4.2 and 6.10 in ISO 15883-1:—, the action level shall be used.

NOTE The analyte values in this document are expressed per unit area (cm^2). Some regional and national guidance specify maximum analyte values per medical device or per medical device side. It is not possible to directly compare numerical values expressed per cm^2 to those values expressed per medical device or per medical device side, unless the surface area of the medical device or medical device side are known, and the appropriate conversion made.

4.4.3.2 Protein assay criteria

The protein assay criteria are:

— Alert level ≥ 3 µg/cm^2

— Action level ≥ 6,4 µg/cm^2

The maximum acceptable level of protein on a cleaned product shall be lower than the action level (see References [42],[51],[72],[76] and[85]), for each sample.

NOTE Protein detection methods can include those specified in Annex C, or as otherwise validated.

4.4.3.3 Assay criteria for other analytes

Other analytes, if used, and their acceptable levels on a cleaned product, include:

a) Total organic carbon (TOC)

1) Alert level ≥ 6 µg/cm^2 (see Reference [85])

2) Action level ≥ 12 µg/cm^2 (see Reference [67])

NOTE TOC is the quantity of carbon present in organic matter and determined as non-purgeable organic carbon.

b) Carbohydrate

1) Alert level ≥ 0,9 µg/cm^2

2) Action level ≥ 1,8 µg/cm^2 (see References [34],[40] and[53])

NOTE The method by Dubois et al.[53] varies in its detection of monosaccharides and therefore the level of detectable carbohydrate, depending on its composition. The alert and action levels provided were based on this limitation.

c) Haemoglobin

1) Alert level ≥ 1,0 µg/cm^2 (see Reference [73])

2) Action level ≥ 2,2 µg/cm^2 (see References [34],[35],[42] and[51])

NOTE Haemoglobin detection methods can include those specified in Annex D, or as otherwise validated.

d) Adenosine triphosphate (ATP)

1) Alert level ≥ 10 femtomoles (fmol) of ATP/cm^2 (see References [37] and[89])

2) Action level ≥ 22 femtomoles (fmol) of ATP/cm^2 (see References [36],[37] and[43])

NOTE Conversion of relative light units (RLU) to femtomoles can be obtained from the specific ATP monitoring equipment supplier.

e) Endotoxin

1) Alert level ≥ 2,2 EU/device (see References [12] and[33])

2) Action level ≥ 20 EU/device (see References [12],[32] and[33])

NOTE 1 Endotoxin is measured in endotoxin units (EU).

NOTE 2 The recommended endotoxin levels are ≤20 EU/device for implants and product that directly or indirectly contact the cardiovascular system and lymphatic system, and ≤2,15 EU/device for a product having intrathecal patient contact (see References [9],[12] and[33]).

NOTE 3 Additional endotoxin studies on medical devices can be found in References [41] and[50].

4.4.4 Process residuals

The cleaning stage(s), including any post-washing rinse(s) shall not leave any process residuals on the load that are potentially harmful during subsequent use or impair the following process stages (see 5.5).

NOTE Refer to process chemicals in ISO 15883-1:—, 4.6.

5 Testing for conformity

5.1 Cleaning test method validation

5.1.1 General

The cleaning test methods employed for both type tests (see 5.3) and performance qualification tests (see 5.4) shall be validated.

NOTE 1 Cleaning test method includes the test soil, soiling method, recovery method, and endpoint analysis.

NOTE 2 ISO/IEC 17025[6] requires test assay methods to be validated. Other references, such as ASTM E-2857,[15] I.C.H guidelines[21], and pharmacopoeias[26],[27],[28],[29], give guidance on requirements for the validation of various types of analytical test methods.

5.1.2 Load soiling method

5.1.2.1 The load soiling method, including its application and any treatment, shall simulate an equivalent challenge to the WD cleaning stage(s) as that presented by worst-case clinically soiled load(s), including the specified load carrier (see 4.2.3 and 5.2.1).

5.1.2.2 The load soiling method shall specify any conditioning, to include drying of soiled product for a defined time, temperature and humidity (i.e. dwell time) that will represent the use of the product being processed in the WD.

NOTE Refer to Köhnlein et al. 2008[62] as an example for conditioning.

5.1.3 Detection method(s)

5.1.3.1 The limit of detection, either directly or by extraction, shall be determined for each analyte and each method used for cleaning validation studies.

5.1.3.2 The test soil recovery efficiency shall be determined. The corresponding correction factor shall be calculated and applied to the results (see 4.2.4).

NOTE ISO 11737-1[2] gives examples of methods of ascertaining bioburden recovery efficiencies and application of bioburden correction factors. These principles and general methods can also be applied regarding test soil recovery.

5.1.3.3 Negative and positive controls shall be conducted to identify interference of process conditions with the detection method (see 5.2.2).

5.1.4 Analyte assay method

The chosen analyte assay method shall be validated for each analyte used for cleaning validation studies.

5.2 Washer-disinfector requirements

5.2.1 The cleaning tests shall be performed on the defined washer-disinfector loads. The worst-case load shall include representative product and specified load carrier(s). Each type of load carrier with representative product shall be tested separately unless a justification is provided to do otherwise.

5.2.2 The WD cleaning stage(s) parameters and cleaning process chemicals shall be specified. Type testing shall be conducted under the specified worst-case parameters (e.g. temperature, time, process chemical concentration, services, pressure, and flow rate).

NOTE Services can include electricity, water, air, and steam supplies.

5.2.3 Cleaning validation shall be tested without any disinfection or drying stage (see ISO 15883-1:—, 6.10).

5.3 Cleaning type test

5.3.1 Principle

In addition to the requirements in this document, the relevant cleaning test requirements of the other applicable parts of ISO 15883 shall apply.

5.3.2 Reagents/materials

Coagulating blood or an alternative test soil meeting the criteria in 4.2 shall be used.

5.3.3 Procedure

5.3.3.1 The test load, washer-disinfector chamber walls and load carrier shall be soiled with test soil (see 4.2.1 and 4.3).

NOTE Refer to Annex A for examples of test soils.

5.3.3.2 Soiled surfaces shall be conditioned as described in 5.1.2.2.

5.3.3.3 Cleaning test stages shall be conducted in triplicate under the worst-case processing conditions as defined for the WD (see 5.2.2).

5.3.4 Acceptance criteria

Cleaning efficacy shall conform with the absence of visible soil as specified in 4.4.2 and with the action levels specified in 4.4.3 for the test load. Where practicable, cleaning efficacy should conform with the

alert levels in 4.4.3 for the test load. Visual examination may be sufficient for chamber walls and load carrier (see 4.4.2).

NOTE 1 The action levels are the acceptance criteria for cleaning efficacy during type testing, but the desired criteria are given as the alert levels.

NOTE 2 Photographic records can assist by capturing visual examination outcomes.

5.4 Cleaning performance qualification test

5.4.1 Principle

Testing shall be conducted in conformance with ISO 15883-1:—, 6.10.3 and the applicable part of ISO 15883.

5.4.2 Reagents/materials

The WD shall be tested using actual loads contaminated by clinical use. These loads shall be representative of those the WD is intended to process and include product that are known as difficult to clean.

For particular product, a surrogate product that simulates clinical use conditions may be used for cleaning efficacy. The use of surrogate product and test soils shall be justified (see 5.1 and 4.2) for applicability to clinical use conditions.

NOTE Surrogate product can be used to simulate product that are difficult to sample without destruction, or for which the extraction efficiency of the sampling method cannot be determined.

5.4.3 Procedure

5.4.3.1 Soiled surfaces shall be held under dwell time conditions representative of worst-case practices (e.g. time, temperature and humidity) prior to cleaning (see 5.1.2.2).

5.4.3.2 Cleaning tests shall be conducted on comparable loads in triplicate (see 5.2).

NOTE Based on a risk analysis, fewer replicates can be justified during requalification.

5.4.4 Acceptance criteria

Cleaning efficacy shall conform with the absence of visible soil specified in 4.4.2 and the action levels specified in 4.4.3 for the test load. Where practicable, cleaning efficacy should conform with the alert levels in 4.4.3 for the test load. Visual examination may be sufficient for chamber walls and load carrier (see 4.4.2).

NOTE Photographic records can assist by capturing visual examination outcomes.

5.5 Process residuals

5.5.1 General

The acceptable amount of process residuals shall be specified as part of risk analysis and cytotoxicity testing as part of type testing. Performance qualification requires periodic sampling of the product for process residuals (see ISO 15883-1:—, Table A.1).

In the case of a change to the process or the process chemicals, type testing and performance qualification shall be repeated.

Compliance with this requirement shall be verified for all process chemicals intended to be used inside the WD.

5.5.2 Risk analysis

A risk analysis shall be documented, demonstrating that the risk of process residuals has been reduced to below harmful levels. The risk analysis shall consider the requirements of ISO 10993-1.

5.5.3 Cytotoxicity

Cytotoxicity tests shall be conducted on medical devices included in the WD load to demonstrate the absence of potentially harmful residuals in conformance with ISO 10993-5 unless otherwise justified as a result of the risk assessment (see 4.4 and 4.6 of ISO 15883-1:—). Any such risk assessment shall include reference to the cytotoxicity of the process chemicals employed during validation. Refer to References [45] and[47].

Additional biocompatibility testing can be required, based on the risk analysis as defined in ISO 10993-1.

5.5.4 Sampling methods

A sampling method for extraction of process residuals from the load and analytical method for detection of process residuals in the samples shall be specified. These methods shall be capable of determining the presence of process chemical(s) at concentrations below that specified as potentially harmful, i.e. as the maximum acceptable (see ISO 15883-1:—, 6.10.4).

NOTE Refer to References [44] and [55].

Sampling shall be done at the end of the complete WD process.

5.5.5 Acceptance criteria

Based on the risk analysis or cytotoxicity test results, an action and alert level shall be specified.

Annex A
(informative)

Examples of test soils

Table A.1 provides condensed versions of examples of test soils that can be used for the testing of cleaning efficacy to meet the requirements of 4.2.

No single test soil has been identified as being representative of all clinical practice.

Table A.1 — Examples of preparation methods for test soils

Examples of procedure	Test soil	Preparation (condensed version)	Reference
General surgery	Coagulated blood	Constituents: — 0,1 ml heparin (10 IU) per 100 ml of sheep blood. Stored at 4 °C to 8 °C and brought to room temperature prior to coagulation. Preparation: — Pour the heparinized blood into a bowl, add protamine sulfate (15 IU) to blood and mix well. — Apply blood immediately to the hinges of the Crile clamp and it coagulates in approximately 10 min to 15 min.	DGKH, DGSV, AKI Guideline [20]
General surgery	Coagulated blood	Constituents: — 3,8 g sodium citrate per 1 000 ml of freshly drawn blood (citrated blood). Stored at 4 °C to 8 °C and brought to room temperature prior to coagulation. Preparation: — Pour the citrated blood into a bowl, add a 250 mmol/l $CaCl_2$ solution to achieve a calcium ion content of 2,5 mmol/l in the final solution. — Apply blood immediately and it coagulates in approximately 10 min to 15 min.	SIS/TR 3:2002 [25]

[a] CIP refers to Collection de l'Institut Pasteur, 25-28 Rue du Dr Roux, 75015 Paris, France. The CIP numbers are the collection numbers of strains supplied by this reference culture collection. This information is given for the convenience of users of this document and does not constitute an endorsement by ISO of the product named.

[b] ATCC refers to American Type Culture Collection, 10801 University Boulevard, Manassas, VA 20110-2209, USA, www.atcc.org. The ATCC numbers are the collection numbers of strains supplied by this reference culture collection. This information is given for the convenience of users of this document and does not constitute an endorsement by ISO of the product named.

NOTE 1 The use of test soils incorporating material from specific animal origin can be restricted by national policies.

NOTE 2 Full details of the test soils provided in Table A.1 and their methods of preparation can be found in the relevant references.

Table A.1 *(continued)*

Examples of procedure	Test soil	Preparation (condensed version)	Reference
General surgery	Two Component Blood Test Soil	Constituents: *Component A:* 400 mg albumin, bovine, protease free; 400 mg haemoglobin, bovine, lyophilized; and 60 mg fibrinogen, bovine, lyophilized. *Component B:* 400 mg albumin, bovine, protease free; 400 mg haemoglobin, bovine, lyophilized; and 12,5 NIH units thrombin, reagent grade from bovine plasma. *Solvent A*—5,0 ml 0,4 % NaCl solution (reagent grade NaCl dissolved in sterile water). *Solvent B*—5,0 ml 0,4 % NaCl solution + 8,0 mmol/l $CaCl_2$ (reagent grade NaCl dissolved in sterile water). Preparation: Dissolve components A and B in their corresponding solvents A and B by shaking for 1 h at room temperature (from 20 °C to 37 °C) in two sealed 10 ml glass vials.	Pfeiffer, M [83]
General surgery, orthopaedic; upper respiratory	Edinburgh soil	Constituents: — 100 ml fresh egg yolk, — 10 ml defibrinated horse or sheep blood, — 2 g dehydrated hog mucin Preparation: — Mix the three constituents together. — Use immediately or store in an airtight container at 2 °C to 5 °C for not more than one week before application to test pieces at room temperature.	HTM 01-01 [22]

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NOTE 1 The use of test soils incorporating material from specific animal origin can be restricted by national policies.

NOTE 2 Full details of the test soils provided in Table A.1 and their methods of preparation can be found in the relevant references.

Table A.1 *(continued)*

Examples of procedure	Test soil	Preparation (condensed version)	Reference
General surgery	Blood Test Soil (BTS)	Constituents: — Bovine albumin: 0,5 g — Bovine haemoglobin: 1,0 g — Sodium alginate: 8,3 mg — Sterile water for irrigation: 27 ml — $CaCl_2$ solution: 15 mM Preparation: Mix dry soil components in a sterile container. Add the sterile water for irrigation and calcium chloride and vortex to homogenize the soil. The components of the soil cross link to form alginate hydrogels. These 3-D protein hydrogels simulate the fibrin hydrogels in clotted blood.	ASTM F3208 [17]
General surgery, orthopaedic	Defibrinated blood soil (Edinburgh soil modified)	Constituents: — 100 ml fresh egg yolk — 100 ml defibrinated sheep blood — 2 g dehydrated hog mucin Preparation: Mix all constituents together using a blender.	ASTM F3208 [17]
Gastrointestinal	Artificial test soil (ATS 2015)	Constituents: — Rosewell Park Memorial Institute (RPMI) 1640 media: 10,4 g; — Heat inactivated bovine calf serum: 100 ml; — (optional) bovine ox gall: 20 g; — Sterile defibrinated sheep blood: 20 % by total volume of final solution; — Sodium bicarbonate: 2 g; — 100 mM sodium pyruvate: 10 ml; — L-Glutamine: 10 ml of 200 mM; and — Total volume 1 l with distilled water.	Alfa & Olson [40]

[a] CIP refers to Collection de l'Institut Pasteur, 25-28 Rue du Dr Roux, 75015 Paris, France. The CIP numbers are the collection numbers of strains supplied by this reference culture collection. This information is given for the convenience of users of this document and does not constitute an endorsement by ISO of the product named.

[b] ATCC refers to American Type Culture Collection, 10801 University Boulevard, Manassas, VA 20110-2209, USA, www.atcc.org. The ATCC numbers are the collection numbers of strains supplied by this reference culture collection. This information is given for the convenience of users of this document and does not constitute an endorsement by ISO of the product named.

NOTE 1 The use of test soils incorporating material from specific animal origin can be restricted by national policies.

NOTE 2 Full details of the test soils provided in Table A.1 and their methods of preparation can be found in the relevant references.

Table A.1 *(continued)*

Examples of procedure	Test soil	Preparation (condensed version)	Reference
		Preparation: Add bovine ox gall, RPMI 1640 medium and sodium bicarbonate to a beaker. Add 700 ml of distilled water and mix well. Then add sodium pyruvate and L-glutamine and again mix well. Pour this mixture into a graduated cylinder. Add distilled water as needed to reach a total volume in the graduated cylinder of 1 l. Filter the entire volume through a 0,22 µm membrane filter into a sterile bottle. Use sterile technique to add sterile sheep blood and calf serum and mix well.	
Flexible endoscopy	Biofilm test soil	Constituents: — *Pseudomonas aeruginosa* (CIP A22)[a] grown on trypticase soy agar. — The test system consists of two polytetrafluoroethylene (PTFE) tubes of 300 mm lengths cut from PTFE tubing, 1,5 m to 2,0 m long and 6 mm inner diameter. In order to ensure that flow is matched, the diameter and length can be matched to the piping in the WD being tested. Preparation: — Inoculate 5 ml to 10 ml *Pseudomonas aeruginosa* bacterial suspension containing about 10^8 colony forming units (CFU) per ml onto PTFE tubing — Connect the two PTFE tubes via isolating valves and Y-piece connectors in place of a section of pipework of the WD. — The test system is maintained in an incubator at (30 ± 2) °C for 72 h to 96 h. — Biofilm is grown on the inner surface of the tubing.	Pineau et al. [86]
Flexible endoscopy	Coagulated blood	Constituents: 0,1 ml heparin per 100 ml of sheep blood. Stored at 4 °C to 8 °C and brought to room temperature prior to coagulation.	Kampf et al. [60] Wehrl & Kircheis [93]

[a] CIP refers to Collection de l'Institut Pasteur, 25-28 Rue du Dr Roux, 75015 Paris, France. The CIP numbers are the collection numbers of strains supplied by this reference culture collection. This information is given for the convenience of users of this document and does not constitute an endorsement by ISO of the product named.

[b] ATCC refers to American Type Culture Collection, 10801 University Boulevard, Manassas, VA 20110-2209, USA, www.atcc.org. The ATCC numbers are the collection numbers of strains supplied by this reference culture collection. This information is given for the convenience of users of this document and does not constitute an endorsement by ISO of the product named.

NOTE 1 The use of test soils incorporating material from specific animal origin can be restricted by national policies.

NOTE 2 Full details of the test soils provided in Table A.1 and their methods of preparation can be found in the relevant references.

Table A.1 *(continued)*

Examples of procedure	Test soil	Preparation (condensed version)	Reference
		Preparation: — Pour heparinized blood into a bowl, add protamine sulfate and mix well. — Blood is applied immediately to the test tubes and coagulates in approximately 10 min to 15 min.	
Human waste containers (toileting)	RAMS	Constituents: — RAMS test soil is a mixture of bovine albumin (0,6 %), mucin (1 %), and native maize starch (3 %). — If pre-tests have shown *Enterococcus faecium* ATCC 6057[b] to be the most resistant bacteria, then use this microorganism as the test organism. — Use sterile stainless-steel plates as test pieces with holes for attachment located at each end for fixing to the devices or to holders, to the load carriers and /or the walls of the WD. The roughened surface (approx. 100 mm x 10 mm) serves as the contamination field. The test pieces should be degreased in a WD or ultrasonic bath. Preparation: — Contaminate test pieces with RAMS, or RAMS with a test microorganism before drying the test pieces. — Fix test pieces onto the load and the WD. — Assess the achieved cleanliness visually to evaluate the cleaning efficacy. — Inspect the test pieces for cleanliness to evaluate the cleaning and the disinfection efficacies. — Transfer the clean test pieces to test tubes containing a growth medium suitable for the test microorganism and calculate the microbial reduction.	DIN 10510 [19] Höller, Krüger & Martiny [30] Krüger & Martiny [66]

[a] CIP refers to Collection de l'Institut Pasteur, 25-28 Rue du Dr Roux, 75015 Paris, France. The CIP numbers are the collection numbers of strains supplied by this reference culture collection. This information is given for the convenience of users of this document and does not constitute an endorsement by ISO of the product named.

[b] ATCC refers to American Type Culture Collection, 10801 University Boulevard, Manassas, VA 20110-2209, USA, www.atcc.org. The ATCC numbers are the collection numbers of strains supplied by this reference culture collection. This information is given for the convenience of users of this document and does not constitute an endorsement by ISO of the product named.

NOTE 1 The use of test soils incorporating material from specific animal origin can be restricted by national policies.

NOTE 2 Full details of the test soils provided in Table A.1 and their methods of preparation can be found in the relevant references.

Annex B
(normative)

Protein-based test soil performance assessment

B.1 Principle

B.1.1 Test background

The test method is based on the test results obtained from an interlaboratory test protocol conducted during the development of this document. The test protocol primarily investigated the dissolution of a defined test soil, under defined temperature and exposure time conditions. Standardized test pieces (STP), emulating the planar surfaces of stainless steel, were coated with freshly coagulating sheep blood as the test soil. The coated STP were immersed in a chemically defined detergent formulation to evaluate the effect of cleaning. Cleanliness was determined by a defined extraction and protein determination method.

B.1.2 Test protocol

This annex specifies the test protocol for evaluating protein-based test soils using a defined test method. Test soils used for conformance with Annex B shall be chosen based on clinical relevance (see 4.2) and shall also meet the acceptance criteria specified in B.2.

The chosen test soil shall contain a minimum amount of protein, regardless of any other components, in order to qualify for assessment and conformance with this Annex.

NOTE 1 Insufficient initial protein in a test soil will not facilitate its measurement at the end of the test.

NOTE 2 Sample results sheets for data entry are provided for guidance in Annex E.

The expected order of protocol execution is as follows:

a) preparation of solutions and reagents;

b) preparation of STPs by cleaning, coating with test soil, conditioning, cutting and weighing;

c) ultraviolet-visible spectroscopy (UV-Vis) ortho-phthalaldehyde (OPA) method for control solutions;

d) preparation of bovine serum albumin (BSA) standard solutions and construction of the calibration curve;

e) UV-Vis OPA method for negative control STPs;

f) UV-Vis OPA method for positive control STPs;

g) testing by immersion, extraction, elution and UV-Vis OPA analysis of processed STPs;

h) final cleaning of all processed STPs.

B.2 Acceptance criteria

The selected test soil (see 4.2.2), shall meet the criteria specified in Table B.1, when tested according to the requirements of this Annex.

The remaining percentage of protein is calculated using Formula (B.3), given in B.11.3.

Table B.1 — Acceptance criteria for tested STP

Test condition	Time	Residual soil remaining on the tested STP
Number 1 Purified water, 25 °C	30 s	Protein remaining shall be: a) equal to or more than 12 %
Number 2 Purified water, 25 °C	90 s	Protein remaining shall be: b) less than test condition #1 AND c) equal to or more than 2 %
Number 3 Purified water, 75 °C	30 s	Protein remaining shall be: d) equal to or more than 12 %
Number 4 Purified water, 75 °C	90 s	Protein remaining shall be: e) less than test condition #3 AND f) equal to or more than 6 %

B.3 STP preparation

B.3.1 General

The material and dimensions for STP and the number of STP required for the test, are specified in B.3.1.1 and B.3.1.2.

B.3.1.1 Number of STPs required

A total of twelve (12) STPs are needed for testing. This consists of:

a) Eight (8) coated STPs - two tests in purified water, at two temperatures and at two (2) time points with two (2) replicates for each test condition;

b) Two (2) coated STPs - positive control testing;

c) Two (2) uncoated STPs – negative control testing.

B.3.1.2 Material and size

The STPs are made from 0,127 mm thick annealed 316L stainless steel foil.

The final size is 50 mm by 50 mm.

B.3.2 Stainless steel preparation

Strips of stainless steel foil shall be cut to 220 mm to 250 mm long x 200 mm wide, cleaned and coated with the test soil using a No. 5 Mayer rod (see Table B.2), and dried. The stainless steel foil is then cut into 50 mm x 50 mm STPs from the centre of the foil (with 25 mm margins for waste/handling on each side) for use in the tests and then conditioned. STPs shall only be used once and all STPs needed for a test series shall be prepared at the same time.

B.3.2.1 Stainless steel foil surface preparation

B.3.2.1.1 Large strips of stainless steel foil, 220 mm to 250 mm long × 200 mm wide, shall be cleaned and verified to be free of protein prior to coating with the test soil.

B.3.2.1.2 The reverse side of the stainless steel foil shall be numbered by scribing and divided into 50 mm x 50 mm STPs for identification (reverse side), using a scriber prior to cleaning as shown in Figure B.1.

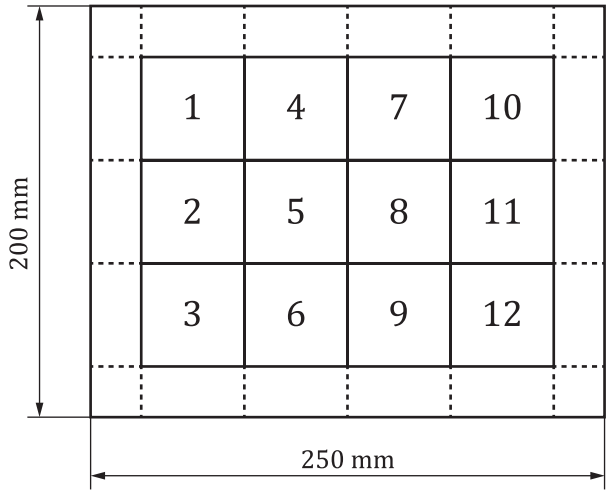


Figure B.1 — Stainless steel foil cut to approximately 250 mm long x 200 mm wide and scribed on one side for identification

B.3.2.2 Cleaning of stainless-steel foil

The scribed stainless-steel foil and all directly associated equipment shall be cleaned according to the following method. Table B.2 lists equipment, and Table B.3 lists chemicals required to perform the cleaning. Equivalent equipment and chemicals with similar specifications may be used, however, the stainless steel shall be identical to that specified in Table B.2.

Table B.2 — Equipment

Equipment	Details	Supplier [a]	Purpose
Annealed stainless steel foil (250 mm x 200 mm)	Finish Type 2R (EN 10088-2, 1.4404)	UK Supplier: Knight Strip Metals Ltd (www.knight-group.co.uk) Code: Annealed 1.4404 (200,0 x 0,127mm, 2R surface finish)	Substrate for STPs
Stainless steel ruler	300 mm long	Not applicable	For marking STPs
Scriber	Not applicable	Not applicable	For scribing stainless steel foil
Scissors	Tailor shears	Faithfull Tools, Stock No. FAI SCTS8, 200 mm size, Long Reach, Galleon Boulevard, Crossways Business Park, Dartford, Kent DA2 6QE, UK, https://www.faithfulltools.com/p/Tailor_Shears	For cutting STPs
Long forceps	Stainless steel (230 mm to 270 mm long)	Not applicable	For holding STPs when tested
Tweezers	Stainless steel	Not applicable	For handling STPs

Table B.2 *(continued)*

Equipment	Details	Supplier [a]	Purpose
Wire wound metering bar (Mayer rod)	No. 5 (K coater, horn coloured) — minimum 250 mm long — close wound — 0,64 mm (0,025 inches) wire diameter — 50 µm (0,0020 inches) wet film deposit	RK PrintCoat Instruments Ltd, Litlington, Royston, Herts, SG8 0QZ, UK	For coating test soil onto stainless steel foil
PVC suction caps	40 mm diameter round button suction cup	Suctioncupsuk http://suctioncupsuk.com	For attaching STP to long forceps
[a] Equipment used for the interlaboratory study was sourced from the suppliers listed. This information is given for the convenience of users of this document and does not constitute an endorsement by ISO of these suppliers.			

Table B.3 — Chemicals required for cleaning stainless steel foil and associated equipment

Chemicals	Grade	CAS No.	Supplier [a]
Decon 90®	-	-	VWR Merck; Cat No. 148-0324 Cat No. 148-0325
Isopropanol	70 % technical	67-63-0	Fisher, Sigma Aldrich, VWR Merck
Purified water	Purified	-	-
[a] Chemicals used for the interlaboratory study were sourced from the suppliers listed. This information is given for the convenience of users of this document and does not constitute an endorsement by ISO of these suppliers.			

B.3.2.3 Procedure

Perform the cleaning of the stainless steel foil separately from the equipment using the same procedure as follows:

a) Place stainless steel foil / equipment for cleaning inside a suitably sized clean plastic container.

b) Prepare a sufficient amount of 5 % w/w solution of Decon 90® cleaning solution in a large beaker.

c) Heat the solution to 45 °C to 50 °C with stirring.

d) Add heated solution to the container ensuring all equipment is fully immersed.

e) Leave to stand for 10 minutes, while occasionally rocking the container.

f) Decant the detergent solution.

g) Rinse equipment twice with purified water while maintaining the rocking motion throughout to ensure all detergent is removed from the equipment.

h) Rinse equipment with isopropanol to facilitate drying.

i) Carefully remove the cleaned equipment and place in a suitable protein-free environment.

B.3.3 STP coating

B.3.3.1 Precautions

Precautions shall be taken to ensure that a defined mass of test soil is applied only to the defined area of the non-scribed side of the stainless steel foil, so as not to cover the scribed side and to ensure the test soil does not flow over the edges of the foil.

NOTE Visual guidance on coating the STPs is available at the following links:

https://www.youtube.com/watch?v=V6-1v0aM-P0

https://www.youtube.com/watch?v=Y-iDF1pKJJA

B.3.3.2 Procedure

Proceed to coat stainless steel foil as follows:

a) Ensure freshly made test soil is used.

b) Lay the stainless-steel foil scribed side down on a non-slip protein free surface.

c) Apply approximately 5 ml of the test soil (see Figure B.2) evenly across one end of the stainless-steel foil (non-scribed side) without going beyond the edges.

d) Spread the test soil with a No. 5 Mayer rod (K coater) with steady and even pressure.

NOTE Automated Mayer rod coaters are available.

e) Allow the coated stainless-steel foil to dry for at least 2 h to 4 h at 20 °C to 25 °C (room temperature).

f) Use clean scissors to carefully cut the coated stainless-steel foil into the individual STPs (approx. 50 mm x 50 mm as scribed in B.3.2.1.2). Cut along the scribed lines (reverse side) whilst avoiding contamination of the coated side.

g) Condition and store the individual STPs at 20 °C to 25 °C and 40 % to 60 % Relative Humidity (RH) for 24 h ± 2 h, under controlled conditions (see B.3.5).

h) Inspect STPs and reject any that are not fully coated.

i) Weigh all STPs on a 4-figure laboratory analytical balance and record the weight [see Table E.4 (positive controls) and Table E.5 (immersion STPs)].

NOTE Clean protein-free plastic disposable petri dishes or similar can be used to weigh and store the STPs, ensuring that the test soil-coated side remains uppermost to prevent protein contamination of the rear of the STPs and that the STPs are covered prior to use.

j) Use coated and weighed STPs within 24 h of conditioning and ensure they are kept under these conditions when not in use.

k) Ensure STPs remain covered during transit and prior to their use.

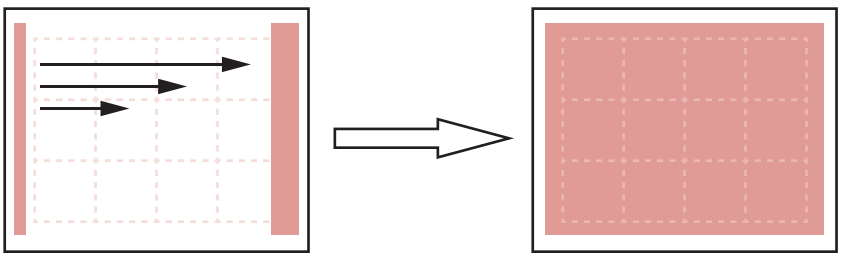


Figure B.2 — Mayer rod direction shown on coated side facing up

B.3.4 Negative control STPs

B.3.4.1 Procedure

Prepare negative control STPs as follows:

a) Scribe a piece of stainless steel foil into two 50 mm x 50 mm STPs and identify as N1 and N2. Cut into the individual STPs and clean (see B.3.2.3).

b) Weigh the individual STPs on a laboratory analytical balance and record the weight (see Table E.3).

NOTE Clean protein-free plastic disposable petri dishes or similar can be used to weigh and store the cut STPs to prevent protein contamination of the STPs.

B.3.5 STP conditioning

The conditioning of the coated STPs shall be conducted between 20 °C to 25 °C and at a relative humidity (RH) between 40 % and 60 %. The conditioning equipment and reagents shall be prepared at least 2 h before coating the stainless-steel foil with test soil.

Condition STPs as follows:

a) Using a suitably sized desiccator or an airtight chamber, add sufficient magnesium nitrate [$Mg(NO_3)_2$] to 100 ml of purified water in a suitably sized vessel with stirring to obtain a saturated solution, ensuring that undissolved magnesium nitrate remains in the vessel.

b) Transfer the saturated solution into a larger vessel or container (e.g. large petri dish) and place it on the bottom of the desiccator/chamber.

c) Secure the desiccator/chamber and close, ensuring it is airtight.

d) Allow at least 2 h for the relative humidity to stabilise.

e) Condition and store the individual STPs at 20 °C to 25 °C and 40 % to 60 % Relative Humidity (RH) for 24 h ± 2 h, under controlled conditions.

B.4 Immersion test apparatus

B.4.1 General

Equipment and accessories required to perform the immersion test are listed below and pictorially represented in Figure B.3.

B.4.2 Apparatus

a) Magnetic stirrer hotplate with a fixed speed setting (50 rpm).

b) Magnetic stirring bar of 25 mm length and 6 mm diameter.

c) Purified water.

d) Glass beaker (400 ml squat form, 80 mm diameter).

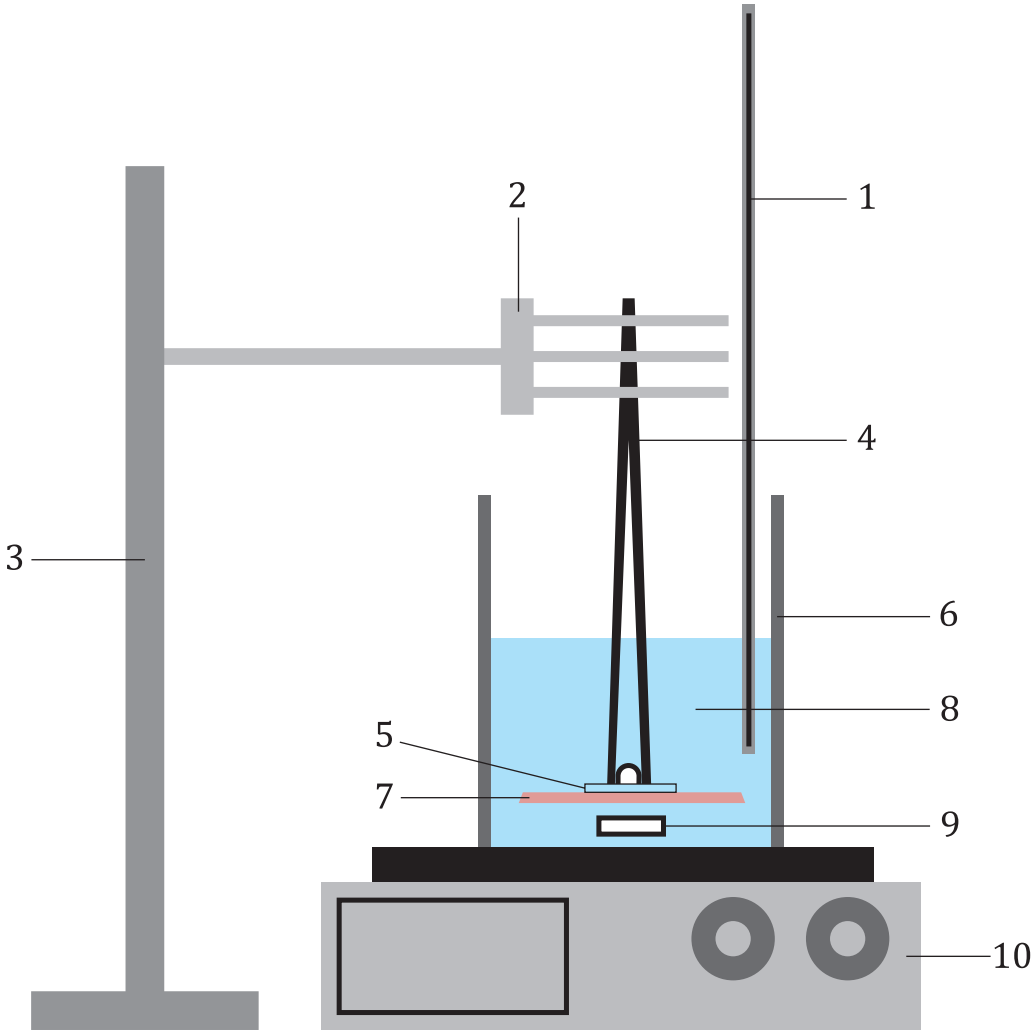
e) Temperature measuring system [(0 - 100) °C ± 0,5 °C] for glass beaker.

f) Retort stand, retort clamps and device to hold PVC suction cap and clamp and STP in position, avoiding direct contact with the coated area.

g) Stopwatch.

h) Long forceps.

i) PVC suction cap.



Key

1 temperature probe or thermometer
2 retort clamp (shown with long forceps)
3 retort stand
4 long forceps
5 PVC suction cap
6 glass beaker
7 STP
8 purified water (equilibrated)
9 magnetic stirrer bar
10 magnetic stirrer hotplate

Figure B.3 — Immersion test apparatus

B.5 Immersion test procedure

B.5.1 General

The immersion test procedure is to be conducted at 2 temperatures, 25 °C ± 2 °C and 75 °C ± 2 °C and at two (2) time intervals of 30 s and 90 s.

B.5.2 Procedure

Proceed with immersion test as follows:

a) Place 100 ml of the purified water in the 400 ml beaker. Adjust the stirrer speed to 50 rpm.

b) Ensure that the temperature in the beaker is at the desired test temperature and within its tolerance of ± 2 °C.

c) Lay the STP soiled side down on a suitably clean and protein free surface and attach a 40 mm PVC suction cap to the non-coated side and fix the long forceps onto the PVC suction cap (see Figure B.4).

d) Suspend the STP in a horizontal orientation in the purified water in the beaker (see Figure B.3) to a depth sufficient to be completely immersed (approximately 10 mm to 15 mm), but also not touching the magnetic stirrer bar, and start the stopwatch.

It is necessary to momentarily turn off the stirrer before immersing the STP to prevent air bubbles forming on the surface of the soiled side of the STP.

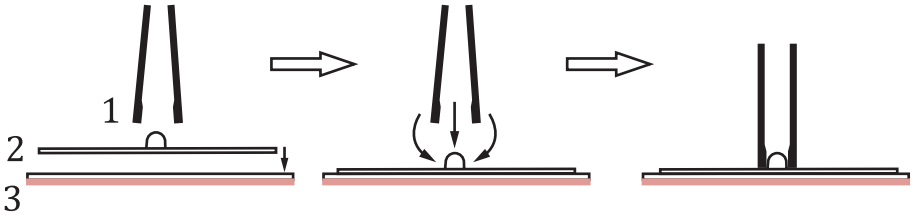
e) After the specified immersion time, stop stirring and remove the clamped STP and gently shake off water test solution.

f) With the STP still attached to the PVC suction cap and secured by long forceps, rest one side of the STP on a protein free absorbent paper towel for 30 s (see Figure B.5).

g) Over the 500 ml glass beaker containing the elution solution and glass beads prepared in B.6.2 use protein-free tweezers to pull the suction release tab on the rim of the suction cap and allow the STP to fall into the elution solution (see Figure B.6).

NOTE The elution procedure is described further in B.6.2.

h) Repeat the immersion test for a total of two (2) replicates per immersion temperature and immersion time



Key

1 forceps/scissor clamp
2 suction cap
3 STP

Figure B.4 — Attaching of long forceps and suction cap to STP

Figure B.5 — Resting one side of STP after immersion for 30 s

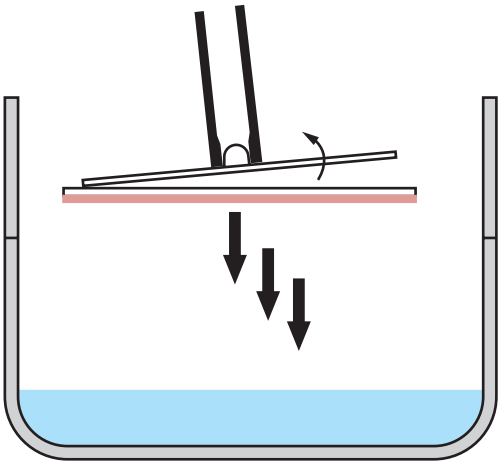


Figure B.6 — Placing STP in elution solution and removing suction cap

B.6 STP elution procedure

B.6.1 General

The elution procedure extracts the residual test soil containing protein into the elution solution, where it is then subsequently analysed by OPA method using UV-Vis spectroscopy.

Protein-free non-latex gloves should be worn to avoid protein contamination.

B.6.2 Procedure

Elute residual protein from STPs as follows:

a) Add 10 ml of the elution solution [i.e. 1 % SDS (sodium dodecyl sulfate) solution, see B.12.2] into a 500 ml beaker.

b) Add 2,0 g of 200 micron to 400 micron glass beads to the beaker.

c) After placing the STP into the elution solution [see B.5.2 g)] cover the beaker to minimise evaporation.

d) Agitate for 20 min to 30 min on an orbital shaker at 20 °C to 25 °C.

NOTE The agitation time is intended to allow sufficient time for all residual protein to be eluted from the STPs. Other test soils might require different times.

e) Stop agitating and remove the STP using protein free tweezers, ensuring that the glass beads are retained in the beaker. Place the STP onto a protein-free absorbent tissue. Retain the STP for determining the STP coat weight by gravimetric analysis (see B.10).

f) Allow the eluted solution to stand until glass beads have settled to the bottom of the beaker. Carefully decant or pipette the elution solution into a vial (e.g. scintillation vial) and cap the contents, ensuring that the glass beads are retained in the beaker. Label the vial with STP number and test conditions, e.g. STP #1, 25 °C, 30 s, Replicate 1.

g) Analyse the eluted solution using the UV-Vis OPA method (see B.9).

B.7 UV-Vis ortho-phthalaldehyde (OPA) method

B.7.1 General

The residual protein level in the eluted solution shall be determined by protein quantification using UV-Vis spectrophotometry directly after extraction using the OPA method. The method shall be used to obtain UV-Vis absorbance readings. Calibration curves and UV-Vis standards/controls are needed for the process.

B.7.2 Equipment

The following equipment and accessories are required to perform the UV-Vis analysis:

a) UV-Vis spectrophotometer set at wavelength of 340 nm;

b) calibrated adjustable pipette;

c) disposable pipette;

d) quartz cuvettes of same type and brand; or high-quality single use cuvettes intended for performing assays over 300 nm, manufactured from scratch-resistant materials, and having the lowest variation in extinction coefficient.

e) appropriate laboratory glassware (e.g. beakers, volumetric flask, etc.).

B.7.3 Calibration curves

For the quantification of complex protein samples, the protein concentration in the test soil shall be assessed against a reference to Bovine Serum Albumin-fraction V (BSA). This allows the quantification of protein on STPs by using a BSA calibration curve.

NOTE The quantification values obtained are only indicative of the protein content as expressed as BSA equivalent.

B.7.4 BSA stock solution preparation

B.7.4.1 General

The BSA calibration curve shall be used to determine the protein content on STPs. The materials needed are listed in Table B.4 and they are used to prepare diluted samples for the calibration curve.

Table B.4 — Chemicals required for BSA stock solution

Reagent	Grade	CAS No.
Bovine serum albumin fraction V (BSA) solid	> 98%	9048-46-8
1 % SDS solution (prepared in B.12.2)	-	-

B.7.4.2 Procedure

Prepare BSA stock solution as follows:

a) Weigh out 100 mg ± 0,1 mg of solid BSA, record the weight, and add to a 100 ml volumetric flask.

b) Add 1 % SDS solution to the flask containing the solid BSA while gently stirring and make up to the 100 ml mark. Ensure complete dissolution is achieved. The stock solution concentration is 1,0 mg/ml (1 000 µg/ml).

NOTE This stock solution is used to make all subsequent dilutions to construct the BSA calibration curve and is intended to be used immediately after preparation.

c) Prepare dilutions from the BSA stock solution using calibrated pipettes, such that an initial concentration range of 2,5 µg/ml to 800 µg/ml are covered (see Table E.2), using 1 % SDS solution as the diluent.

The concentration should not exceed 800 µg/ml (+ 20 µg/ml), to maintain calibration curve linearity.

d) Dilutions shall be kept at 20 °C to 25 °C (room temperature) away from light when not in use.

B.7.5 UV-Vis system and reagent compatibility for the OPA method

B.7.5.1 General

Prior to analysis, it is necessary to determine if the reagents and diluents are suitable for use to minimise erroneous results. There are four (4) controls that shall be used to determine if the equipment (spectrophotometer and cuvettes) are acceptable and two (2) reagent controls to determine if the absorbances obtained during testing will be reliable. This ensures:

a) diluents used are acceptable (i.e. not degraded),

b) absorbance measured are normalised for calculations, taking into account absorbances from the reagents and diluents used to prepare samples.

B.7.5.2 System suitability controls

B.7.5.2.1 The four (4) system suitability controls shall be performed before and after the UV-Vis spectrophotometer is used for the analysis. The controls are listed in Table B.5 along with the method and typical absorption values.

Table B.5 — System suitability tests

Control	Method
Air	— Obtain three (3) readings from the UV-Vis spectrophotometer (at 340 nm) without a cuvette in the instrument. Do not zero the instrument before and after each reading. This is the 'baseline zero' sample. If the values obtained are not between - 0,001 and + 0,001, only then perform an "Auto-Zero" on the instrument. Cuvettes in some cases can give readings > 0,001. — Any absorbance by a cuvette when used for subsequent analysis cancels out when sample calculations are performed. — Typical absorption: – 0,001 to 0,001 (or 0,000 on auto-zero)
1 % SDS solution (prepared in B.12.2)	— Fill a cuvette with 1 % SDS solution only and obtain three readings from the solution. — Typical absorption: 0,03 to 0,05
SDS/borate solution (prepared in B.12.6)	— Fill a cuvette with SDS/borate solution only and obtain three readings from the solution. — Typical absorption: 0,03 to 0,05
OPA reagent (prepared in B.12.7)	— Fill a cuvette with OPA reagent only and obtain three readings from the solution. — Typical absorption: 0,09 to 0,11 and shall be ≤ 0,15

B.7.5.2.2 For each control, obtain three (3) values before constructing the BSA calibration curve (see B.7.6) and record the mean (see Table E.1).

It is considered standard practice to use the same cuvette for the system suitability and reagent controls (B.7.5.3). However, where this is not feasible, measures should be taken to avoid intra-cuvette inconsistency and cuvette to cuvette variance.

B.7.5.2.3 The cuvette shall be rinsed twice with 1 % SDS solution prior to each spectrophotometric analysis.

B.7.5.3 Reagent controls

B.7.5.3.1 The two reagent controls shall be performed before and after the UV-Vis spectrophotometer is used for the analysis. The controls are listed in Table B.6 along with the method and typical absorption values.

Table B.6 — Reagent control tests

Reagent control	Method
Reagent Control A	— Fill a cuvette with equal volumes of 1 % SDS solution and SDS/borate solution (1:1 v/v ratio) using a calibrated automated pipette. Mix the contents in the cuvette using a disposable pipette. — Allow to stand between 60 s and 90 s then obtain three (3) readings at 340 nm. — Typical absorption: 0,03 to 0,05
Reagent Control B	— Fill a cuvette with equal volumes of 1 % SDS solution and OPA reagent (1:1 v/v ratio) using a calibrated automated pipette. Mix the contents in the cuvette using a disposable pipette. — Allow to stand between 60 s and 90 s then obtain three (3) readings at 340 nm. — Typical absorption: 0,06 to 0,09

B.7.5.3.2 For both controls, obtain three (3) values before constructing the BSA calibration curve (see B.7.6) and record the mean (see Table E.1).

B.7.5.3.3 The cuvette shall be rinsed twice with 1 % SDS solution prior to each spectrophotometric analysis.

If the absorbance of Reagent Control A solution is greater than the absorbance of Reagent Control B in the same session the solutions shall be prepared again.

B.7.6 Constructing a BSA calibration curve

The following procedure is for a cuvette of nominal volume of 3,0 ml. Adjust the volumes accordingly if the nominal volume of a cuvette is greater or smaller than this value. Equal volumes of sample and diluent are used (1:1 v/v ratio mixture). Multiple cuvettes for determining sample absorptions are acceptable provided that all cuvettes used give similar absorbance for the reagent used.

B.7.6.1 Procedure for sample preparation and UV data acquisition

Prepare sample to acquire data as follows:

a) Ensure that the system suitability controls (see B.7.5.2) and reagent controls (see B.7.5.3) have been performed on the same day before progressing.

   Do not auto-zero the spectrophotometer at any stage.

b) Using a calibrated automated pipette, add equal volumes of Reagent Control A and the prepared dilution of BSA being tested (e.g. 1,50 ml of Reagent Control A and 1,50 ml BSA dilution) to the cuvette.

c) Mix the contents of the cuvette using a disposable pipette. Care should be taken to minimise the formation of air bubbles as this can interfere with the spectrophotometer reading.

d) Allow to stand for between 60 s and 90 s and ensure that the sample is not turbid/hazy.

e) Place cuvette in UV-Vis spectrophotometer and obtain three readings from the solution. Record the mean value (see Table E.2).

   NOTE The absorbance obtained is for the BSA sample/self-absorbance at the diluted concentration used.

f) Remove cuvette, discard contents and rinse twice with 1 % SDS solution.

g) Using a calibrated automated pipette add equal volumes of OPA reagent and the prepared dilution of BSA being tested (e.g. 1,50 ml of OPA reagent and + 1,50 ml BSA dilution) to the cuvette.

h) Mix the contents of the cuvette using a disposable pipette. Care should be taken to minimise the formation of air bubbles as this can interfere with the spectrophotometer reading.

i) Allow to stand for between 60 s and 90 s and ensure that the sample is not turbid/hazy.

j) Place cuvette in UV-Vis spectrophotometer and obtain three readings from the solution. Record the mean value (see Table E.2).

   NOTE The absorbance obtained is for the BSA sample/OPA reagent at the diluted concentration used.

k) Remove cuvette, discard contents and rinse twice with 1 % SDS solution.

l) Repeat B.7.6.1 b) to B.7.6.1 k) for all BSA dilutions prepared to cover 2,5 µg/ml to 800 µg/ml range.

NOTE The concentration of BSA solution sample (BSA initial concentration) is effectively halved when diluted equally with diluent in the cuvette (referred to as the Normalised BSA Concentration).

B.7.6.2 Corrected Normalised Mean Sample Absorbances ($^{N}E_{BSA}$)

For each BSA dilution sample used (n), the mean respective absorbances are calculated, and then normalised through subtraction using the reagent controls (see Table E.2).

a) Normalised BSA sample/self-absorbance ($^{SA}E_n$) is Mean Absorbance Value - Mean Reagent Control A

b) Normalised BSA sample/OPA reagent ($^{OPA}E_n$) is Mean Absorbance Value - Mean Reagent Control B

c) Corrected Normalised Mean Sample Absorbance ($^{N}E_n$) is $^{OPA}E_n$ - $^{SA}E_n$

Where 'n' is the BSA initial concentration in specified µg/ml for the sample being tested.

B.7.6.3 Plotting the BSA calibration curve

Plot the BSA calibration curve as follows:

a) A plot of Normalised BSA concentration (µg/ml, x-axis) against Corrected Normalised Mean Sample Absorbances ($^{N}E_{BSA}$, y-axis) shall be plotted and a line of best fit using linear trend fitting (of the type y = m*x* + c) shall be constructed.

b) The plot slope (*x* -coefficient term), the graph intercept (c-intercept term), and the R-squared value (R^2 required to be > 0,990), shall be determined and recorded (see Table E.1). The graph terms shall be used to calculate the concentration of protein of the test soil in the test samples for negative, positive and immersion tested STPs.

NOTE The c-intercept can be a non-zero value if the plot does not go through the origin of the graph.

B.8 Negative & positive controls

B.8.1 Negative control STPs

Blank determinations with uncoated STPs shall be conducted to establish the background level of any interfering substances. Ensure the weight of both STPs used as negative control STPs (N1, N2) are determined and recorded prior to testing. Negative control STPs protein determination shall be conducted after construction of the BSA calibration curve.

STP extraction using the elution method is completed followed by determination of the amount of protein (as BSA equivalent) present on both individual STPs using the OPA method to obtain the Corrected Normalised Mean Sample Absorbance (^{N}E) and then utilising the graphical terms derived from the BSA calibration curve.

The limit of detection shall be the absorbance obtained for Reagent Control A (see B.7.5.3).

B.8.1.1 Measuring the protein content of negative control STPs

Measure the protein content of negative control STPs as follows:

a) Complete the STP extraction by the elution method (see B.6). Retain the STP for gravimetric analysis (see B.10).

b) Ensure that the system suitability controls (see B.7.5.2) and reagent controls (see B.7.5.3) have been performed on the same day before progressing.

   Do not auto-zero the spectrophotometer at any stage.

c) Using a calibrated automated pipette add equal volumes of Reagent Control A and extracted negative control STP elution solution (1,50 ml each) to the cuvette.

d) Mix the contents of the cuvette using a disposable pipette. Care should be taken to minimise the formation of air bubbles as this can interfere with the spectrophotometer reading.

e) Allow to stand between 60 s and 90 s and ensure the sample is not turbid/hazy.

f) Place sample in UV-Vis spectrophotometer and obtain three readings for the solution. Record the mean value (see Table E.3).

   NOTE The absorbance obtained is for the negative control sample/self-absorbance.

g) Remove the cuvette, discard contents and rinse twice with 1 % SDS solution.

h) Using a calibrated automated pipette add equal volumes of OPA reagent and the extracted negative control STP elution solution (1,50 ml each) to the cuvette.

i) Mix the contents of the cuvette using a disposable pipette. Care should be taken to minimise the formation of air bubbles as this can interfere with the spectrophotometer reading.

j) Allow to stand for between 60 s and 90 s and ensure that the sample is not turbid/hazy.

k) Place cuvette in UV-Vis spectrophotometer and obtain three readings from the solution. Record the mean value (see Table E.3).

l) Remove cuvette, discard contents and rinse twice with 1 % SDS solution.

m) Repeat B.8.1.1 a) to B.8.1.1 l) for the other negative control STP.

NOTE The absorbance obtained is for the negative control sample/OPA reagent.

B.8.1.2 Corrected Normalised negative control Sample Absorbances

B.8.1.2.1 For each negative control sample, the mean respective absorbances are calculated and then normalised through subtraction using the reagent controls (see Table E.3).

a) Normalised sample/self-absorbance (${}^{SA}E_n$) is Mean Absorbance Value – Mean Reagent Control A

b) Normalised sample /OPA Reagent (${}^{OPA}E_n$) is Mean Absorbance Value – Mean Reagent Control B

c) Corrected Normalised Mean Sample Absorbance for negative control STP: ${}^{N}E_n$ is ${}^{OPA}E_n$ - ${}^{SA}E_n$

Where '*n*' is 1 or 2 with respect to STP N1 or N2.

B.8.1.2.2 The amount of protein (mg) for each negative control STP tested is calculated using the following Formula (B.1), where the terms *x* (coefficient) and *c* (intercept) have been previously determined by the BSA calibration curve:

$$\left(\frac{{}^{N}E-c}{x}\right)\div 50 \qquad \text{(B.1)}$$

The mean protein content for the negative control STPs are then calculated (see Table E.3).

B.8.2 Positive control STPs

Ensure the weight of both STPs used as positive control STPs are determined and recorded prior to testing [see B.3.3.2 i)]. Positive control STPs protein determination shall be conducted after the construction of the BSA calibration curve.

STP extraction using the elution method is completed followed by determination of the amount of protein (as BSA equivalent) present on both individual STPs using the OPA method to obtain the Corrected Normalised Mean Sample Absorbance (${}^{N}E$) and then utilising the graphical terms derived from the BSA calibration curve.

The mean protein content from both positive control STPs shall be calculated and then used in conjunction with the mean STP coat weight (see B.10) to determine the fractional protein content per soil weight (mg protein/mg soil coated). This value is used to calculate theoretical coating of protein on coated STPs used in the immersion tests.

The limit of detection shall be the absorbance obtained for Reagent Control A (see B.7.5.3).

B.8.2.1 Measuring the protein content of positive control STPs

Measure the protein content of the positive control STPs as follows:

a) Complete the STP extraction by the elution method (see B.6). Retain the STP for gravimetric analysis (see B.10). In addition to the scribed STP number, label the elution contents as P1 and P2.

b) Ensure that the system suitability controls (see B.7.5.2) and reagent controls (see B.7.5.3) have been performed before progressing.

Do not auto-zero the spectrophotometer at any stage.

c) Using a calibrated automated pipette add 1 000 µl of the positive control STP eluted solution to a vial containing 4 ml (4 000 µl) of 1 % SDS solution and mix well. This is a necessary dilution step required to ensure absorbance values obtained are within the linear range of the BSA calibration curve.

d) Using a calibrated automated pipette add equal volumes of Reagent Control A and the diluted sample [see B.8.2.1 c)] of 1,50 ml each to the cuvette.

e) Mix the contents of the cuvette using a disposable pipette. Care should be taken to minimise the formation of air bubbles as this can interfere with the spectrophotometer reading.

f) Allow to stand between 60 s and 90 s and ensure the sample is not turbid/hazy.

g) Place sample in UV-Vis spectrophotometer and obtain three readings for the solution. Record the mean value (see Table E.4).

NOTE The absorbance obtained is for the positive control sample/self-absorbance.

h) Remove the cuvette, discard contents and rinse twice with 1 % SDS solution.

i) Using a calibrated automated pipette add equal volumes of OPA reagent and the diluted sample [see B.8.2.1 c)] of 1,50 ml each to the cuvette.

j) Mix the contents of the cuvette using a disposable pipette. Care should be taken to minimise the formation of air bubbles as this can interfere with the spectrophotometer reading.

k) Allow to stand for between 60 s and 90 s and ensure that the sample is not turbid/hazy.

l) Place cuvette in UV-Vis spectrophotometer and obtain three readings from the solution. Record the mean value (see Table E.4).

NOTE The absorbance obtained is for the positive control sample/OPA reagent.

m) Remove cuvette, discard contents and rinse twice with 1 % SDS solution.

n) Repeat B.8.2.1 a) to B.8.2.1 m) for the other positive control STP.

B.8.2.2 Corrected Normalised positive control Sample Absorbances ($^{N}E_{n}$)

B.8.2.2.1 For each positive control sample used, the mean respective absorbances are calculated and then normalised through subtraction using the reagent controls, such that:

a) Normalised sample/self-absorbance ($^{SA}E_{n}$) is Mean Absorbance Value – Mean Reagent Control A

b) Normalised sample /OPA Reagent ($^{OPA}E_{n}$) is Mean Absorbance value – Mean Reagent Control B

c) Corrected Normalised Mean Sample Absorbance for positive control STP; ($^{N}E_{n}$) is $^{OPA}E_{n}$ - $^{SA}E_{n}$

Where '*n*' is 1 or 2 with respect to STP P1 or P2 as identified in B.8.2.1 a).

B.8.2.2.2 The amount of protein (mg) for each sample tested is given by the following Formula (B.2), where the terms *x* (coefficient) and *c* (intercept) have been previously determined by the BSA calibration curve:

$$\left(\frac{^{N}E-c}{x}\right)\div 10 \qquad \text{(B.2)}$$

The mean protein content for the positive control STPs is then calculated (see Table E.4).

B.9 Determination of residual protein for immersion tested STPs – UV-Vis analysis procedure

For STPs that have undergone the immersion testing (see B.5) followed by the STP extraction by elution (see B.6), the eluted solution can require additional dilution prior to UV-Vis analysis if the sample absorbance is greater than 1,200 [see B.9 b)]. Determine the residual protein by UV-Vis analysis as follows:

a) Follow the method for measuring the protein content of negative control STPs (see B.8.1.1) and calculate the residual protein content using Formula (B.1) (see B.8.1.2.2).

b) If the absorbance is found to be greater than 1,200, follow the method for measuring the protein content of positive control STPs (see B.8.2.1) that includes the additional dilution step and calculate the residual protein content using Formula (B.2) (see B.8.2.2.2).

NOTE The limit of detection is the absorbance obtained for the Reagent Control A (see B.7.5.3).

c) Record results (see Table E.5).

B.10 Determination of STP coat weight

B.10.1 Cleaning of processed STPs

All STPs used in the study shall be thoroughly cleaned, dried and weighed to determine the actual test soil coat weight of the STP.

B.10.2 Procedure

Determine the STP coat weight as follows:

a) Add 300 ml SDS cleaning solution (see B.12.3) into a clean glass beaker (500 ml) and warm to 40 °C.

b) Remove any loose glass beads from the STPs.

c) Add all the STPs to the solution.

d) Agitate the solution continuously for 10 min to 15 min and ensure STPs do not stick together.

e) Remove a single STP using tweezers and submerge into a beaker containing 500 ml purified water whilst swirling the STP, then submerge into a second clean beaker containing 500 ml purified water and swirl again.

f) Remove the STP using tweezers and submerge into a beaker containing 100 ml acetone or 70 % isopropanol and swirl solution.

g) Remove the STP using tweezers and place on a lint free absorbent cloth or tissue and allow to air dry on each side.

h) Inspect the STP for cleanliness (using tweezers to handle the STP). If particulates/residues are visible, repeat B.10.2 a) to B.10.2 h).

i) Repeat B.10.2 e) to B.10.2 h) for all retained STPs.

B.10.3 STP coat weight (gravimetric analysis)

Conduct the gravimetric analysis as follows:

a) Using clean and dry tweezers or forceps, weigh individually all retained STPs that have been thoroughly cleaned and dried (see B.10.2) on a 4-figure laboratory analytical balance and record the weight (see Tables E.3, E.4, and E.5).

b) Calculate the STP coat weight by subtracting the cleaned STP weight [see B.10.3 a)] from the weight of the STP determined in B.3.3.2 i) (positive control and immersion STPs) or in B.3.4.1 b) (negative control). See Tables E.3, E.4 and E.5.

B.11 Calculation of protein remaining on STP following the immersion procedure

B.11.1 Calculate the fractional protein content per soil weight using the following calculation and record results (see Table E.4):

Mean Positive Control Protein Content (see B.8.2.2.2) ÷ Mean Positive Control Coat Weight (see B.10.3)

B.11.2 Calculate the theoretical calculated protein content (mg) of a given STP used for the immersion testing using the following calculation and record results (see Table E.6):

STP_n coat weight (see B.10.3) x fractional protein content per soil weight (see B.11.1)

Where 'n' is the scribed STP number.

B.11.3 The percentage of protein remaining on a tested STP is calculated using Formula (B.3) as follows:

$$\left(\frac{\text{Protein Remaining on a given STP (see B.9)}}{\text{Theoretical Calculated Protein Content (see B.11.2)}} \right) \text{x } 100 \qquad \text{(B.3)}$$

B.11.4 The percentage of remaining protein calculated from Formula (B.3) shall conform with the acceptance criteria specified in B.2.

B.12 Preparation of solutions

B.12.1 General

Solutions prepared below, in particular those containing SDS as a component occasionally become turbid, typically below 20 °C or on extended storage. Prior to use, all solutions shall be inspected for precipitation. If precipitation is observed, warm solutions to 35 °C under warm tap water and stir until all solids dissolve.

Prepared solutions, especially those used to conduct UV-Vis controls (see B.7.5) shall be clear and free from cloudiness.

B.12.2 Preparation of 1 % SDS solution

The 1 % SDS solution (see Table B.7) shall be used as an elution solution for immersion of processed STPs and as both the diluent and cuvette rinse solution for UV-Vis analysis. The solution is also used as a UV-Vis control (system suitability test). The method below is for 2,0 l which is typically sufficient for the testing. The solution is stable at 20 °C to 25 °C (room temperature) and requires no special storage.

Table B.7 — Components required for 1 % SDS solution

Reagent	Grade	CAS No.
Sodium dodecyl sulfate (SDS)	> 99,0 %	151-21-3
Purified water	-	-
0,1 M Sodium hydroxide solution (NaOH)	Volumetric analysis grade	1310-73-2

Prepare 1 % SDS solution as follows:

a) Weigh out 20,000 g ± 0,002 g of SDS and record the weight.

b) Into a 2 l beaker, add 1 800 ml of purified water.

c) Add the SDS to the purified water and stir until a solution is obtained.

d) Measure and adjust pH to pH 11,0 to 11,1 using sodium hydroxide solution if required.

e) Transfer the solution into a 2 l volumetric flask.

f) Rinse the 2 l beaker with 150 ml of purified water and add washings to the volumetric flask.

g) Make up to the 2 l mark on the volumetric flask with more purified water.

h) Seal the volumetric flask and mix contents.

i) Transfer the contents of the 2 l volumetric flask into a suitable bottle.

j) Label the bottle with preparation date and contents.

B.12.3 Preparation of processed STPs cleaning solution

The 10 % w/w SDS (sodium dodecyl sulfate) solution (see Table B.8) is used in the cleaning of processed STPs prior to determining the coat weight (see B.10). The method below is for 2,0 l, which is typically sufficient for the cleaning. The solution is stable at 20 °C to 25 °C (room temperature) and requires no special storage.

Table B.8 — Components required for 10 % (w/w) SDS solution

Reagent	Grade	CAS No.
Sodium dodecyl sulfate (SDS)	> 99,0 %	151-21-3
Purified water	-	-

Prepare STP cleaning solution as follows:

a) Weigh out 200,0 g ± 0,5 g of SDS and record the weight.

b) Into a 2 l beaker, add 1 800 g ± 0,5 g of purified water (by weight).

c) Add the SDS to the purified water and stir until a solution is obtained. Heat to 50 °C if required to aid dissolution.

d) Transfer into a suitable bottle and label with contents and preparation date.

e) The solution is stable at 20 °C to 25 °C (room temp.) and requires no special storage.

B.12.4 Preparation of 20 % (w/w) SDS solution

The 20 % (w/w) SDS (sodium dodecyl sulfate) solution (see Table B.9) is used as a component to prepare the SDS/borate (see B.12.6) and the OPA Reagent (see B.12.7). The method below is for 200 ml. The solution is stable at 20 °C to 25 °C (room temperature) and requires no special storage.

Table B.9 — Components required for 20 % (w/w) SDS solution

Reagent	Grade	CAS No.
Sodium dodecyl sulfate (SDS)	> 99,0 %	151-21-3
Purified water	-	-

Prepare 20 % (w/w) SDS solution as follows:

a) Add 140 ml purified water into a 200 ml volumetric flask.

b) Weigh out 40,00 g ± 0,02 g of SDS and record the weight.

c) Add the SDS, portion wise into the volumetric flask while stirring gently to aid dissolution. (Use heat up to 50 °C if required).

d) Once dissolved, add purified water and make up to the 200 ml mark.

e) Stopper the volumetric flask and mix the contents gently to minimise foaming.

f) Transfer the contents of the volumetric flask into a suitable bottle and label contents.

Preparation of the 20 % (w/w) SDS solution causes the solution to foam upon agitation. Mixing should be kept to minimum but sufficient to aid dissolution of solids.

B.12.5 Preparation of 0,1 M borate solution

The 0,1 M borate solution (see Table B.10) is used as a component to prepare the SDS/borate (see B.12.6) and the OPA Reagent (see B.12.7). The solution is stable at 20 °C to 25 °C (room temperature) and requires no special storage.

Table B.10 — Components required for 0,1 M borate solution

Reagent	Grade	CAS No.
Sodium tetraborate decahydrate	> 99,5 %	1303-96-4
Purified Water	-	-

Prepare 0,1 M borate solution as follows:

a) Weigh out 76,20 g ± 0,01 g of sodium tetraborate decahydrate and record the weight.

b) Add 1 500 ml of purified water (that has been warmed to 40 °C to 45 °C) into a 2 l beaker.

c) Add the sodium tetraborate decahydrate to the beaker containing the purified water with stirring until dissolved.

d) Transfer the solution into a 2 l volumetric flask.

e) Rinse out the 2 l beaker with 3 x 100 ml purified water and add washings to the 2 l volumetric flask.

f) Allow the contents of the 2 l volumetric flask to equilibrate to 20 °C to 25 °C (room temperature).

g) Make up to the 2 l mark with purified water, stopper and mix contents.

h) Transfer the 0,1 M borate solution into a bottle and label contents and preparation date.

B.12.6 Preparation of SDS/borate solution

The SDS/borate solution (see Table B.11) is used as a diluent to determine the sample self-absorbance of test samples and for constructing the BSA calibration curve (see B.7.6). The solution is also used as a UV-Vis control (system suitability test). The method below is for 1,0 l and the solution is stable at 20 °C to 25 °C (room temperature) and requires no special storage.

Table B.11 — Components required for SDS/borate solution

Reagent	Grade	CAS No.
20 % (w/w) SDS solution (prepared in B.12.4)	-	-
0,1 M borate solution (prepared in B.12.5)	-	-
Methanol (MeOH)	> 99,0 %	67-65-1
Purified water	-	-
Sodium hydroxide 1 M solution	> 99,0 %	-

Prepare SDS/borate solution as follows:

a) Into a 1 l volumetric flask add in order with minimum agitation

   1) 50 ml of 20 % (w/w) SDS solution

   2) 20 ml of methanol

   3) 20 ml of purified water

b) Carefully add 0,1 M borate solution to the 1 l volumetric flask and make up to the 1 l mark.

c) Stopper the flask and mix the contents, then transfer the contents into a suitable bottle and label with contents and preparation date.

d) Adjust the pH of the solution to 9,3 ± 0,1 using 1 M sodium hydroxide solution.

B.12.7 Preparation of OPA reagent

The OPA reagent (see Table B.12) is used as a diluent to determine the sample - OPA absorbance of test samples and diluted samples prepared for constructing the BSA calibration curve. The reagent is also a UV-Vis control (system suitability test). The method below is for 1,0 l. Store the OPA reagent at 20 °C to 25 °C (room temperature) in the dark until required for use and use within 14 days.

Table B.12 — Components required for OPA reagent

Reagent	Grade	CAS No.
ortho-Phthalaldehyde (OPA)	> 99,0 %	643-79-8
2-Mercaptoethanesulfonic acid, sodium salt	> 98,0 %	19767-45-4
20 % (w/w) SDS solution (prepared in B.12.4)	-	-
0,1 M borate solution (prepared in B.12.5)	-	-
Methanol (MeOH)	> 99,0 %	67-56-1
Purified water	> 99,0 %	-
Sodium hydroxide 1 M solution	> 99,0 %	-

Prepare OPA reagent solution as follows:

a) Weigh 1,6 g ± 0,01 g of OPA and 4,64 g ± 0,005 g of 2-mercaptoethanesulfonic acid sodium salt into separate vials and record the weights.

b) Add 20 ml of methanol to the OPA and dissolve the solid, warming briefly under hot purified water to aid dissolution if required.

c) Add 20 ml of purified water to 2-mercaptoethanesulfonic acid sodium salt and dissolve the solid.

d) Into a 1 l beaker, add 50 ml of 20 % (w/w) SDS solution and 750 ml of 0,1 M borate solution and stir the mixture gently.

e) Add the OPA/methanol solution [see B.12.7 b)] to the beaker and rinse vial with 20 ml of 0,1 M borate solution. Add rinses to the beaker.

f) Add the 2-mercaptoethanesulfonic acid sodium salt solution [see B.12.7 c)] to the beaker and rinse vial through with 20 ml of 0,1 M borate solution. Add rinses to the beaker.

g) Cover the beaker and continue to stir until all solids dissolve and a complete solution is obtained.

h) Transfer the contents of the beaker into a 1 l volumetric flask.

i) Rinse out the beaker twice using 50 ml of 0,1 M borate solution each time and add rinses to the flask.

j) Make up to the 1 l mark with 0,1 M borate solution; stopper and mix the contents.

k) Transfer the solution into a suitable sized bottle (amber glass preferred).

l) Adjust the pH of the solution to 9,3 ± 0,1 using 1 M sodium hydroxide solution.

m) Wrap the outer bottle with aluminium foil (including base and neck) to avoid degradation of the solution.

Annex C
(informative)

Examples of test methods for the detection and assessment of residual proteinaceous contamination

C.1 General

The initial examination of cleaning efficacy during type testing (as well as performance qualification and routine testing) is carried out by visual inspection. A quantitative evaluation of residual protein should only be carried out for visually clean product. The choice of the quantitative detection method should consider its detection range, accuracy and specificity and should be appropriate for the acceptance criteria and the product being tested. The modified ortho-phthalic dialdehyde (OPA) method (see References [57], [74] and [75]) and the bicinchoninic acid (BCA) method (see References [90] and [92]), are preferred for protein quantification after sampling. Since the OPA and BCA methods do not measure protein directly, residual process chemicals or other substances can interfere with the protein determination. Testing a negative control (i.e. an unsoiled medical device processed and extracted identically to the test devices) is required to rule out interferences.

Other protein assay methods can be used provided they are validated.

C.1.1 Sampling

Samples for residual protein analysis are obtained by rinsing (or extracting) the product or targeted areas of the product with an aqueous solution of 1 % sodium dodecyl sulfate (SDS).

Preferably, extraction should be limited to the areas of the product that come into contact with the patient tissue and pose a risk for transfer during reuse. In this way results from non-critical areas of product can be avoided.

The 1 % SDS solution used for extraction should be adjusted to pH 11. pH test strips with a graduation of at least 0,5 pH units, or a pH meter should be used to adjust the pH with 0,1 N sodium hydroxide solution. The efficacy of the protein extraction method should be checked by ensuring that all residues are dissolved, e.g. by staining non-solubilized residues using total protein stains such as Coomassie R-250, Amido black, SYPRO Ruby, STAINS ALL, Ponceau S, etc.

NOTE 1 Insoluble proteins in the extract are not effectively detected by the photometric detection methods described.

The extraction should be carried out with a minimal volume of 1 % SDS solution to avoid any analytical errors that arise from using large extraction volumes and to have a suitable detection limit to assess the attainment of the residual protein-surface acceptance criterion.

Large extraction volumes can significantly reduce the total sensitivity by dilution effects. Therefore, the extraction volume should be as small as possible while making sure that all soiled surface areas are constantly wetted or submersed, allowing for movement of the extraction fluid on the sampled surface.

Nevertheless, very small extraction volumes can reduce efficiency of protein recovery. As a result, careful balancing of rinsing volume can be required. It is more important for the product to be wetted than to reduce the extraction volume below a given limit. Further, repeated extractions with a smaller volume can be more efficient than a single extraction with a larger volume. Protein extraction can be

increased by using warm SDS solution (e.g. 40 °C) and mechanical support by sonication if staining indicates nonsolubilized residues.

NOTE 2 Published data in Reference [94] show that hot SDS-solution at pH = 11 could be needed to dissolve high molecular weight fibrin residues derived from blood soil.

It is not possible to use a photometric measurement for protein determination on any turbid solution without correction using filtration or a separate photometric measurement of turbidity. The cause of turbidity should be determined and remediated if turbidity results from an error in reprocessing. Turbid samples can be filtered to remove cloudiness using a 0,2 µm syringe filter, however, it is essential to ensure that the filter membrane does not bind protein. Membrane filtration should be validated along with all other steps in protein determination.

Extraction with the SDS solution should be carried out over an established period of time with intervals of intensive mixing (either by hand or using a vortexer) or flushing. For example, SDS extraction could be carried out by soaking for 30 min with intensive mixing/flushing performed for 30 s every 10 min. Other validated extraction methods can also be used. Extraction methods should ensure appropriate extraction efficiency values.

EXAMPLE 1 Extraction from the surfaces of a product in a polypropylene bag with 2 ml to 5 ml of SDS solution.

Extraction of residual contamination on an entire product can be performed with 2 ml to 5 ml 1 % SDS solution in a sealed, stable polypropylene (PP) bag large enough to hold the product. Once the product is sealed in the bag with 1 % SDS solution, extraction is achieved by inverting or shaking the bag and manipulating the product through the bag. Hinged product should be operated through the bag to enable contact of the SDS solution with the hidden surfaces of the hinge.

NOTE 3 Polypropylene, other bag materials and plastic tubes are often treated with slip agents or mold release agents (e.g. eurcamide) that can be released from the surface of the plastic during intensive mixing with the product and produce turbidity in the extraction solution. It is good practice to test a negative control (i.e. an unsoiled product processed and extracted identically to the test product) to identify any interference.

Extraction is also possible for product with lumens and large, easily accessible cavities, (e.g. trocar sleeves) in a suitable polypropylene bag. The SDS solution can be directed through these cavities by tilting back and forth. Depending on the product design, the bag should also be turned so that all the internal areas come into contact with the extraction solution.

EXAMPLE 2 Partial extraction from a hinged product with 2 ml to 3 ml of SDS solution.

Some hinged product (e.g. Crile clamps) are used in standardized testing to evaluate cleaning efficacy in critical functional areas, such as the hinge. During extraction in SDS solution, the hinge requires intensive movement of the joint.

EXAMPLE 3 Extraction from a shaft tube with 2 ml to 5 ml SDS solution.

Product with narrow lumens can be placed with the distal end in a beaker and held upright with a laboratory stand clamp. Extraction is carried out by flushing 2 ml to 5 ml of the SDS solution through the product lumen with a pipette or a syringe. The SDS solution is drawn up from the beaker. Recirculate the same SDS solution five (5) times through the lumen during the extraction procedure at intervals (e.g. 5 min).

An analogous extraction procedure is also possible by separating the shaft from the insert with the functional end for minimally invasive surgical (MIS) product that can be disassembled, and by placing the insert in a section of tubing with a length and inner diameter just large enough to contain the product. Clamp or seal one end and add the SDS solution for extraction. Also clamp or seal the other end and rotate the product so that the SDS solution flows back and forth.

Product with internal channels that potentially come into contact with contaminated material and cannot be visually inspected should be flushed with SDS solution for sample collection.

C.1.2 Calibration of protein detection method used

The accurate quantification of residual protein is dependent upon the preparation of the protein to be used as the reference standard for the method. The standard protein used for calibration is bovine serum albumin (BSA, fraction V). Prepare or purchase a BSA standard stock solution with a concentration of 2,00 mg/ml. Use this stock solution to prepare serial dilutions of various concentrations (e.g. 200 µg/ml, 100 µg/ml, 50 µg/ml, 25 µg/ml, 12,5 µg/ml, 6,25 µg/ml) diluted in 1 % SDS solution and stored in clean tubes. The amount of protein in the product extract is determined by comparing the extract measurement with the standard curve created by a regression line calculated from the measurement of the standard dilutions of known concentration. The standard and extract samples should be analysed in the same way.

C.2 Modified OPA method for the assessment of residual protein contamination

C.2.1 Principle

The modified *ortho*-phthalic dialdehyde (OPA) test method is a quantitative method for the determination of primary (1°) amino groups, which are present in protein at the amino terminus of the peptide (α-) and on the amino acid lysine (ε-). OPA, in the presence of N,N-dimethyl-2-mercaptoethyl-ammonium chloride and a primary amino group, forms stable fluorescent alkylthio-2-alkylisoindoles which is detected spectrophotometrically at 340 nm. Primary amines are ubiquitous in nature and can be present in process chemicals used in processing, therefore, negative controls are included to identify possible interfering substances.

NOTE Methods with similar or higher accuracy e.g. fluorometer can be used (see Reference [82]).

C.2.2 Procedure

To prepare the OPA reagent solution, dissolve 40 mg *ortho*-phthalic dialdehyde in 1 ml methanol (mix until completely dissolved), then add 50 ml 0,1M disodium tetraborate buffer (pH 9,3), 100 mg N,N-dimethyl-2-mercaptoethyl-ammonium chloride and 1,25 ml of an aqueous 20 % SDS solution. Store the solution in an opaque bottle to protect it from light and use it only for one working day.

Prepare a spectrophotometer blank by diluting OPA reagent with 1 % SDS solution at the same proportion used for the protein determinations of the standard solutions and product extracts. Use the blank value to zero the spectrophotometer. Adjust the spectrophotometer to measure at 340 nm and use 1 cm path length quartz cuvettes (or disposable half-micro cuvettes suitable for this wavelength).

When measuring the standard solutions and the product extract samples, the same proportion of sample and OPA reagent are used. For example, add 200 µl of the standard solution or device extract sample to 1 ml (1:5) of freshly prepared OPA reagent solution in the cuvette. Proportions of sample to OPA reagent down to 1:1 can also be used as long as the pH of the reaction mixture is maintained at 9,3 (confirmed using a pH test strip). Lower dilutions can cause a change in the pH of the OPA reagent and potentially invalidate the results. Mix the reaction solution with a pipette or cover the cuvette with a cap and mix it thoroughly by gentle inversion. Measure the absorbance after 5 min incubation time and after any bubbles from mixing have moved to the surface of the reaction mixture.

When absorbance values are > 0,010, the analysed samples might contain substances that absorb light at 340 nm. Consequently, the self-absorbance should be determined and subtracted from the OPA measurement result. The self-absorbance is measured by using the same ratio of sample to 1% SDS solution (instead of OPA reagent solution), i.e. pipette 200 µl of the 1 % SDS product extract in 1 ml of 1 % SDS solution, mix and measure the absorbance against the 1 % SDS solution. This is subtracted from the absorbance measured for the sample after OPA reaction and the protein amount determined relative to the calibration with the bovine serum albumin dilution series (see C.1.2).

C.2.3 Calculation of the protein content

To calculate the total residual protein on the product or extracted portion of the product, the volume of SDS solution used in the extraction is taken into account. The concentration of protein in the extract

sample (in µg/ml) is multiplied by the total volume of 1 % SDS used to extract the product/portion of the tested product (in ml).

For example, if the measured concentration of protein is 10 µg/ml and the extract volume is 5 ml, then the total residual protein is 50 µg. As mentioned previously, unnecessarily high extraction volumes will lead to a multiplication of both the protein results and the analytical noise and interfering effects to the protein determination method.

C.2.4 Acceptance criteria/interpretation of results

Consult the pertinent literature regarding interfering substances.

C.3 BCA method for the assessment of residual protein contamination

C.3.1 Principle

The bicinchoninic acid test method is similar to the Biuret reaction in which protein causes the reduction of copper (II) ions (Cu^{2+} or cupric form) in the reagent to copper (I) ions (Cu^{+} or cuprous form) in alkaline environment. The cuprous ion is then chelated with two (2) molecules of bicinchoninic acid (BCA) resulting in a violet chromophore with a strong light absorption at 562 nm.

Other substances, which can reduce copper from the cupric to the cuprous form under the conditions of the test, will also result in the formation of the BCA chromophore. Testing a negative control is useful to rule out interferences.

NOTE 1 Commercial test kits are available.

NOTE 2 For references, see Smith et al.[90] and Stoschek[92].

C.3.2 Materials/solutions

Prepare reagent A and B as follows:

Reagent A: 1 g sodium bicinchoninate, 2 g sodium carbonate, 0,16 g sodium tartrate, 0,4 g NaOH and 0,95 g sodium bicarbonate, brought to 100 ml distilled water and adjusted to pH 11,25 with 10 M NaOH.

Reagent B: 0,4 g cupric sulfate (pentahydrate) in 10 ml distilled water.

These reagents are used to prepare the standard working solution (SWS) by mixing 100 parts of reagent A with two (2) parts of reagent B. Store the SWS solution in an opaque bottle to protect it from light and use it only for one working day.

C.3.3 Procedure

For the assay, use 100 µl product extract or standard solution and 1 ml SWS (or 200 µl extract or standard and 2 ml SWS) and prepare a blank control with 100 µl purified water plus 1 ml SWS. Mix and allow to react for 2 h at room temperature (20 °C ± 1 °C). Then measure the blank control, BSA standards and the product extract samples within a 10 min time period with the photometer set at 562 nm, reset to zero by measuring purified water in a clean cuvette. Adjust the measurement value by subtracting the blank value from the measured value.

NOTE The BCA test is a rate reaction (has no true end point); consequently, the colour continues to develop until the substrates (Cu II and BCA, supplied in excess) are consumed. No significant deviation in chromophore arises within 10 mins.

The protein concentration of the product extract is determined relative to the calibration with the bovine serum albumin standards (see C.1.2).

C.3.4 Calculation of the total protein content

Calculate the total protein of the product extracts (see C.2.3).

C.3.5 Acceptance criteria/interpretation of results

Consult the pertinent literature regarding interfering substances.

Annex D
(informative)

Examples of test methods for the detection of haemoglobin for the assessment of cleaning efficacy

D.1 Haemoglobin detection methods

D.1.1 Principle

In addition to the initial examination of cleaning efficacy by visual inspection, residual haemoglobin testing can be performed in addition to residual protein testing. The use of residual haemoglobin detection can be justified by the nature of soils that are being evaluated i.e. soils are expected to contain haemoglobin. The choice of method should consider its detection range, accuracy and specificity and should be appropriate for the acceptance criteria and the product being tested. All reactions in the chemical quantification of haemoglobin occur with the non-protein haeme component, whose centrally located iron molecule is the site of oxygen binding.

Since the quantification methods described below do not measure haemoglobin directly (or measure directly at a specific wavelength), residual process chemicals or other substances can interfere with the haemoglobin reaction or might absorb at the same wavelength. Testing a negative control (i.e. an unsoiled product processed and extracted identically to the test product) is useful to rule out chemical or optical interferences.

NOTE For references, see Huy et al.[59], Michels[73], and Stadie[91].

D.1.2 Sampling

Samples for residual haemoglobin analysis are obtained by rinsing (or eluting) or swabbing the product or targeted areas of the product with an aqueous solution of 1 % sodium dodecyl sulfate (SDS), pH 11.

See C.1.1 for details on the methods recommended for the extraction of residual protein, which apply equally to haemoglobin.

D.2 Semi-quantitative detection of haemoglobin using microhematuria test strips

D.2.1 Principle

Commercially available reagent test strips for detecting microhematuria are extremely sensitive and provide a simple way to obtain quick, semi-quantitative results for residual haemoglobin assessment and can be used as an initial screening test. These products do not require special equipment, produce a result in minutes and have a limit of detection (LOD) of < 1 µg/ ml.

These test strips use a chemistry based on the pseudoperoxidase activity of haemoglobin and are especially useful as a screening test to confirm the absence of haemoglobin on a reprocessed surgical device, since the LOD is very low compared to the allowed limit for residual haemoglobin. For example, if the test product has a surface area of 100 cm^2, then the allowed residual haemoglobin is 220 µg at the action level of 2,2 $µg/cm^2$.

D.2.2 Procedure

a) Place a drop of product extract (see D.1.2) on the test area at the end of the strip and wipe (from the side of the strip) or tap any excess extract off the pad.

b) Hold the test strip in a horizontal position for 60 s.

c) Hold the test area against the colour blocks on the side of the bottle and carefully match the colour of the test area to the corresponding colour block on the bottle.

d) Read the test area between 1 min and 2 min.

D.2.3 Acceptance criteria/interpretation of results

If haemoglobin is detected by a colour change exceeding the levels in 4.4.3.3 c), then further investigation should be conducted using a quantitative method.

D.3 Quantitative detection of haemoglobin using a spectrophotometer

D.3.1 General

Several methods for the spectrophotometric measurement of haemoglobin are available which are easy to use and provide good accuracy and sensitivity. Methods depend either, on the pseudoperoxidase activity of haemoglobin or the conversion of oxyhaemoglobin to various oxidation products with defined absorption spectra. Examples are shown in D.3.2 and D.3.5.

D.3.2 Calibration and calculation of haemoglobin concentration

The accurate quantification of residual haemoglobin is highly dependent upon the preparation of the calibrator used as the standard for the method. Preparations of human or bovine haemoglobin can be used as the standard for calibration. Carefully prepare or purchase a certified human or bovine haemoglobin standard stock solution. Use this stock solution to prepare serial dilutions of various concentrations (e.g. 200 µg/ml, 100 µg/ml, 50 µg/ml, 25 µg/ml, 12,5 µg/ml, 6,25 µg/ml) diluted in 1 % SDS solution and stored in clean tubes. The amount of haemoglobin in the device extract is determined by comparing the extract absorbance measurement with the standard curve created by a regression line calculated from the measurement of the standard dilutions of known concentration. The standard and extract samples should be analysed in the same way.

D.3.3 Calculation of total residual haemoglobin

To calculate the total residual haemoglobin on the product or extracted portion of the product, the volume of SDS solution used in the extraction should be taken into account. The concentration of haemoglobin in the extract sample (in µg/ml) is multiplied by the total volume of 1 % SDS used to extract from the product /portion of the tested product (in ml).

For example, if the measured concentration of haemoglobin is 10 µg/ml and the extract volume is 5 ml, then the total residual haemoglobin is 50 µg. Unnecessarily high extraction volumes will lead to a multiplication of both the haemoglobin results and the analytical noise and interfering effects to the haemoglobin determination method.

Consult the pertinent reference literature[59],[73],[91], regarding interfering substances.

D.3.4 Drabkin's Reagent

The method using the Drabkin's Reagent is mentioned only for historical purpose owing to its widespread use as the reference method for haemoglobin measurement in clinical blood samples. This method is accurate and specific for this purpose, but has disadvantages which make it unsuitable for use in the assessment of cleaning efficacy, including a high detection limit of 30 mg/ml (30 000 µg/ml) and an environmental toxicity hazard related to the disposal of cyanide compounds in the reagent.

In this reaction, oxyhaemoglobin is oxidized to methaemoglobin by potassium ferricyanide. Methaemoglobin then reacts with potassium cyanide to form a stable cyanomethaemoglobin with a

broad absorption peak measured at 540 nm. The reaction is shown below with the non-reactive ions omitted.

$$HbFe(II) + (Fe(III)(CN)_6)^{3-} \rightarrow HbFe(III) + (Fe(II)(CN)_6)^{4-}$$

$$HbFe(III) + CN^- \rightarrow HbFe(III)CN$$

The results are calibrated against a certified cyanomethaemoglobin standard.

D.3.5 Methods based on the pseudoperoxidase activity of haemoglobin

D.3.5.1 General

Haemoglobin (heme) has peroxidase activity and catalyzes the oxidation of 3,3',5,5'-tetramethylbenzidine (TMB) in the presence of a peroxide to form the blue-green chromophore TMB diamine which has absorbance maxima at 370 and 652 nm. Commercial TMB reagents with different sensitivities are commonly available. These commercial reagents use an organic hydroperoxide (e.g. cumene hydroperoxide) in place of hydrogen peroxide, resulting in a single reagent that is stable at 4 °C for months. The basic reaction is shown in Figure D.1 along with the structures of the peroxide substrate used in a typical commercial assay kit.

$$TMB + HOO^- \rightarrow TMB\ diamine\ (chromophore) + HO^-$$

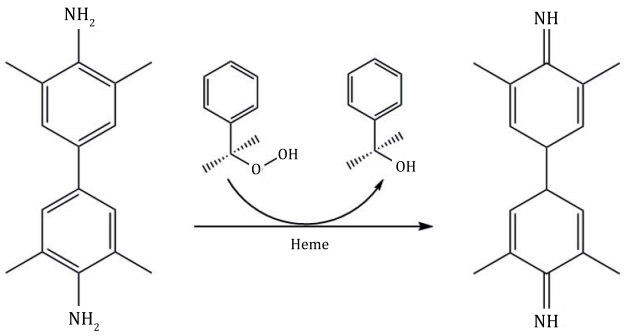


Figure D.1 — Example of a typical haemoglobin assay kit reaction

The TMB assay is a rate reaction, which continues until the substrate is depleted. Consequently, the TMB diamine chromophore should be measured in each standard solution and product extract, exactly at a specified incubation time (during the linear phase of the reaction) to provide meaningful analytical results. The TMB reaction is well suited for use in a plate reader or an automated spectrophotometer where the timing of the absorbance measurements is controlled. A standard spectrophotometer can also be used if care is taken in the timing of the measurements.

D.3.5.2 Procedure

a) Prepare a solution of 0,1 % TMB in 5 % acetic acid.

b) Prepare a separate solution of 3 % hydrogen peroxide.

c) The activated TMB reagent is prepared by mixing 1 ml of the 0,1 % TMB solution with 4 drops of 3 % hydrogen peroxide (or larger volumes using the same proportions). The activated TMB solution is not stable and should be made fresh for each day. Alternatively, use a stable commercial TMB reagent.

d) Add 200 µl of each haemoglobin standard solution and product extract sample into clean test tubes.

e) To each sample, add 800 µl of the activated TMB reagent or a commercial TMB reagent (equilibrated to room temperature) to each tube and mix gently.

f) Allow the samples to incubate for precisely 30 min at room temperature.

g) The TMB reaction can be quenched by the addition of 1 ml of 2 M sulfuric acid at the end of the incubation period. This produces a stable (endpoint) yellow chromophore with an absorbance maximum of 450 nm. The quenching step cannot be used for standard samples or product extracts containing 1 % SDS. In the presence of both sulfuric acid and the TMB reagent, SDS forms an insoluble precipitate, which causes turbidity in the reaction mixture and invalidates the spectrophotometric measurement.

h) Perform the spectrophotometric measurements of each sample at 652 nm according to the principles described in C.2.2.

Annex E
(informative)

Test soil performance assessment – Sample results sheets

Tables E.1 to E.6 are examples of a format that can be used for data entry and calculations for the test soil performance test specified in Annex B.

Table E.1 — System suitability and reagent controls

Control sample type (see B.7.5.2 & B.7.5.3)	Expected range		Unacceptable absorbance values	Mean absorption value
	Low	High		
Air	-0,001	+0,001	> 0,003	
1 % SDS	0,03	0,05	> 0,08	
SDS/borate solution	0,03	0,05	> 0,08	
OPA reagent	0,08	0,11	> 0,15	
1 % SDS solution / SDS/borate solution (Reagent Control A)	0,03	0,05	> 0,08	
1 % SDS solution / OPA reagent (Reagent Control B)	0,06	0,09	> 0,15	

Table E.2 — Construction of BSA calibration curve

BSA dilutions			BSA sample/self-absorbance		BSA sample/OPA reagent absorbance		Corrected Normalised Mean Absorbance (see B.7.6.2) $^{N}E = {}^{OPA}E - {}^{SA}E$
BSA stock solution required (ml) (for 10 ml volumetric standard)	Initial concentration (µg/ml)	Normalised BSA concentration (µg/ml)	Mean value [see B.7.6.1 e)]	Normalised ^{SA}E (see B.7.6.2)	Mean value [see B.7.6.1 j)]	Normalised ^{OPA}E (see B.7.6.2)	
0,025	2,50	1,25					
0,050	5,00	2,50					
0,100	10,00	5,00					
0,200	20,00	10,00					
0,400	40,00	20,00					
0,800	80,00	40,00					
1,000	100,00	50,00					
2,000	200,00	100,00					
3,000	300,00	150,00					
4,000	400,00	200,00					
5,000	500,00	250,00					
8,000	800,00	400,00					
Graphical plot axis		X-axis values	Plot BSA calibration curve: Plot 'X' against 'Y' axis values allowing c-intercept to be a non-zero value. Determine the c-intercept and *x*-coefficient (slope) of the line.				Y-axis values
c-Intercept value (8 dp)			*x*-coefficient (8 dp)			R^2 value >0,990	
NOTE Ensure system suitability and reagent controls (Table E.1) have been completed on the same day as constructing the BSA calibration curve.							

Table E.3 — Protein content measurement of negative controls

STP number	Coated STP weight (g) [see B.3.4.1 b)]	STP weight after cleansing (g) [see B.10.3 a)]	Coat weight (mg) [see B.10.3 b)]	UV-Vis spectrometry absorbance raw data (see Table E.1)		Mean SA [see B.8.1.1 f)]	Mean OPA [see B.8.1.1 k)]	UV-Vis spectrometry - corrected data			Protein content on STP (mg)
				Mean RC(A)	Mean RC(B)			^{SA}E *Mean SA – Mean RC(A)*	^{OPA}E *Mean OPA – Mean RC(B)*	^{N}E $^{OPA}E - {}^{SA}E$	*Use Formula (B.1) below*
N1											
N2											
Mean Coat Weight (mg)								Mean Protein Content (mg)			

RC(A) = Reagent Control A	SA = Sample/self-absorbance	^{SA}E = Normalised Mean Sample/self-absorbance	Protein Content Formula (B.1) for negative control STPs
RC(B) = Reagent Control B	OPA = Sample/OPA reagent absorbance	^{OPA}E =Normalised Mean Sample/OPA Reagent absorbance	$\left(\frac{{}^{N}E - c}{x}\right) \div 50$ *c*-intercept and *x*-coefficient terms are derived from the BSA calibration curve
The protein content for a given coat weight maybe negative due to operations in the construct of the BSA calibration curve.		^{N}E = Corrected Normalised Mean Absorbance	

NOTE Ensure system suitability and reagent controls (Table E.1) have been completed on the same day as measuring the protein content of negative controls.

Table E.4 — Protein content measurement of positive controls

STP number	Coated STP weight (g) [see B.3.3.2 i)]	STP weight after cleansing (g) [see B.10.3 a)]	Coat weight (mg) [see B.10.3 b)]	UV-Vis spectrometry absorbance raw data (see Table E.1)		Mean SA [see B.8.2.1 g)]	Mean OPA [see B.8.2.1 l)]	**UV-Vis spectrometry - corrected data**			**Protein content on STP (mg)**
				Mean RC(A)	Mean RC(B)			$^{SA}\mathbf{E}$ *Mean SA – Mean RC(A)*	$^{OPA}\mathbf{E}$ *Mean OPA – Mean RC(B)*	$^{N}\mathbf{E}$ $^{OPA}E - {}^{SA}E$	Use Formula (B.2) below
(P1)											
(P2)											
Mean Coat Weight (mg)								Mean Protein Content (mg)			
Fractional Protein Content Per Soil Weight (see B.11.1) →				$\left(\frac{\text{Mean Protein Content (mg)}}{\text{Mean Soil Coat Weight (mg)}}\right)$							

RC(A) = Reagent Control A	**SA** = Sample/self-absorbance	$^{SA}\mathbf{E}$ = Normalised Mean Sample/self-absorbance	Protein Content Formula (B.2) for positive control STPs $\left(\frac{{}^{N}E - c}{x}\right) \div 10$ *c*-intercept and *x*-coefficient terms are derived from the BSA calibration curve
RC(B) = Reagent Control B	**OPA** = Sample/OPA Reagent absorbance	$^{OPA}\mathbf{E}$ =Normalised Mean Sample/OPA Reagent absorbance	
The protein content formula takes into account the additional dilution performed in B.8.2.1 c)		$^{N}\mathbf{E}$ = Corrected Normalised Mean Absorbance	

NOTE Ensure system suitability and reagent controls (Table E.1) have been completed on the same day as measuring the protein content of positive controls.

Table E.5 — Protein content measurement of immersion tested STPs

Immersion Temperature			**°C**		**Immersion Time**		**seconds**			
STP number	Coated STP weight (g) [see B.3.3.2 i)]	STP weight after cleansing (g) [see B.10.3 a)]	Coat weight (mg) (see B.10.3 b)	UV-Vis spectrometry absorbance raw data (See Table E.1)		Mean SA (see B.9)	Mean OPA (see B.9)	**UV-Vis spectrometry - corrected data**			**Protein content on STP remaining (mg)**
				Mean **RC(A)**	Mean **RC(B)**			^{SA}**E**	^{OPA}**E**	^{N}**E**	
								Mean SA – Mean RC(A)	*Mean OPA – Mean RC(B)*	$^{OPA}E - {}^{SA}E$	*Use the appropriate Protein Content* Formula (B. 1) or (B.2) *below*

RC(A) = Reagent Control A	**SA** = Sample/self-absorbance	**Protein Content Formula (B.1)**	**Protein Content Formula (B.2)**
RC(B) = Reagent Control B	**OPA** = Sample/OPA Reagent absorbance	$\left(\frac{^{N}E - c}{x}\right) \div 50$.	$\left(\frac{^{N}E - c}{x}\right) \div 10$

^{SA}**E** = Normalised Mean Sample/self-absorbance	^{OPA}**E** =Normalised Mean Sample/OPA Reagent absorbance	^{N}**E** = Corrected Normalised Average absorbance

If sample absorbance is ≤ 1,200 no additional dilution is required and Protein Content Formula (B.1) is used.

If sample absorbance is > 1,200 additional dilution is required [see B.8.2.1 c)] and Protein Content Formula (B.2) is used.

NOTE Ensure system suitability and reagent controls (Table E.1) have been completed on the same day as measuring the protein content of immersion tested STPs.

Table E.6 — Calculation of protein remaining on STP following the immersion procedure

Immersion temperature		**°C**	**Immersion time**		**seconds**
A	B	C	D	E	**F**
STP number	STP coat weight (mg) (see Table E.5)	Fractional protein content per soil weight (see Table E.4)	Theoretically calculated protein content before immersion test (see B.11.2) **B x C**	Protein content on STP remaining (see Table E.5)	% protein remaining (see B.11.3) *Formula (B.3)* (E / D) x 100

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NATIONAL ANNEX F

(*National Foreword*)

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