



Space product assurance

Microbial examination of flight hardware and cleanrooms

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This Standard is one of the series of ECSS Standards intended to be applied together for the management, engineering and product assurance in space projects and applications. ECSS is a cooperative effort of the European Space Agency, national space agencies and European industry associations for the purpose of developing and maintaining common standards.

Requirements in this Standard are defined in terms of what shall be accomplished, rather than in terms of how to organize and perform the necessary work. This allows existing organizational structures and methods to be applied where they are effective, and for the structures and methods to evolve as necessary without rewriting the standards.

The formulation of this Standard takes into account the existing ISO 9000 family of documents.

This Standard has been prepared by the ECSS-Q-70-55A Working Group, reviewed by the ECSS Executive Secretariat and approved by the ECSS Technical Authority.

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Change log

Firs issue.

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Introduction

The UN Outer Space Treaty of 1967 sets up the general principles applicable to the exploration and use of outer space. Article IX of the Outer Space Treaty constitutes the primary statement of international law: “States parties shall pursue studies of outer space, including the Moon and other celestial bodies, and conduct exploration of them so as to avoid their harmful contamination and also adverse changes in the environment of the Earth resulting from the introduction of extraterrestrial matter and, when necessary, adopt appropriate measures for this purpose”. Harmful contamination in that sense is defined as biological contamination, including organic-constituents, to protect the environment in order to allow future exobiology research. The Committee On Space Research (COSPAR) has established some planetary protection guidelines, based on the Outer Space Treaty. These guidelines impose requirements on spaceflight missions according to target body/mission type combinations.

The objective of this Standard is to ensure that the proper procedures for establishing the microbiological contamination on flight hardware and controlled environments are in place to meet the planetary protection constraints.

1. Scope

This standard defines the test procedures for detecting microbiological contamination on surfaces in cleanrooms, cleanroom air, and flight hardware using culture-dependent and culture-independent microbiological assays.

The following test methods are described:

- Surface and air sampling and detection of biological contaminants with swabs, wipes, contact plates and air samples, followed by cultivation for bioburden determination.
- Sampling of biological contaminants by DNA analysis from swabs and wipes.

The test methods described in this standard apply to controlling the microbiological contamination on all manned and unmanned spacecraft, launchers, payloads, experiments, ground support equipment, and cleanrooms with planetary protection constraints.

This standard does not address molecular contamination control.

This standard does not address the principles and basic methodology for controlling cleanrooms and associated controlled environments with constraints on particulate contamination.

2. Normative references

The following dated normative documents are called by the requirements of this ECSS Standard and therefore constitute requirements to it. Subsequent amendments to, or revisions of any of these publications do not apply.

NOTE However, parties to agreements based on this ECSS Standard are encouraged to investigate the possibility of applying the most recent editions of the normative documents indicated below.

ECSS-P-001	ECSS – Glossary of terms
ECSS-P-00	Standardization Policy
ECSS-P-001	Glossary of terms
ECSS-Q-00	Space product assurance - Policy and principles
ECSS-Q-20	Space product assurance - Quality assurance
ECSS-Q-70-01	Space product assurance - Cleanliness and contamination control
ISO 14644 part 1	Cleanrooms and associated controlled environments - Part 1: Classification of air cleanliness
ISO 14644 part 2	Cleanrooms and associated controlled environments - Part 2: Specifications for testing and monitoring to prove continued compliance with ISO 14644-1

3.

Terms, definitions and abbreviated terms

3.1. Terms defined in other standards

For the purpose of this Standard, the terms and definitions from ECSS-P-001B apply, in particular for the following terms:

3.2. Terms specific to the present standard

3.2.1. **bioburden**

quantity of viable microorganisms that can survive a heat shock of 80°C for 15 min measured with a specified assay

3.2.2. **biodiversity**

identification of type of micro-organism, measured with specified assays

3.3. Abbreviated terms

The following abbreviations are defined and used within this standard:

Abbreviation	Meaning
ASTM	American Society for Testing and Materials
DNA	Desoxyribonucleinacid
DNase	Deoxyribonuclease
IEST	Institute of Environmental Sciences and Technology
IPA	Isopropylakcohol
ISO	International Organization for Standardization
NASA	National Aeronautics and Space Administration
PBS	Phosphate-buffered saline solution
PCR	Polymerase chain reaction
PDA	Potato Dextrose Agar
R2A	A low nutrient bacterial medium with agar
rDNA	Ribosomal DNA
RNase	Ribonuclease
S	Svedberg units
TE	Tris-EDTA, 2-Amino-2-(hydroxymethyl)propane-1,3-

TSA

diol ethylenediaminetetraacetic acid

Tryptic Soy Agar

4. Principles

The activities related to microbial examination requirements, specifications, procedures and reports are described in Figure 1, and the related standardization requirements are captured in clause 5.

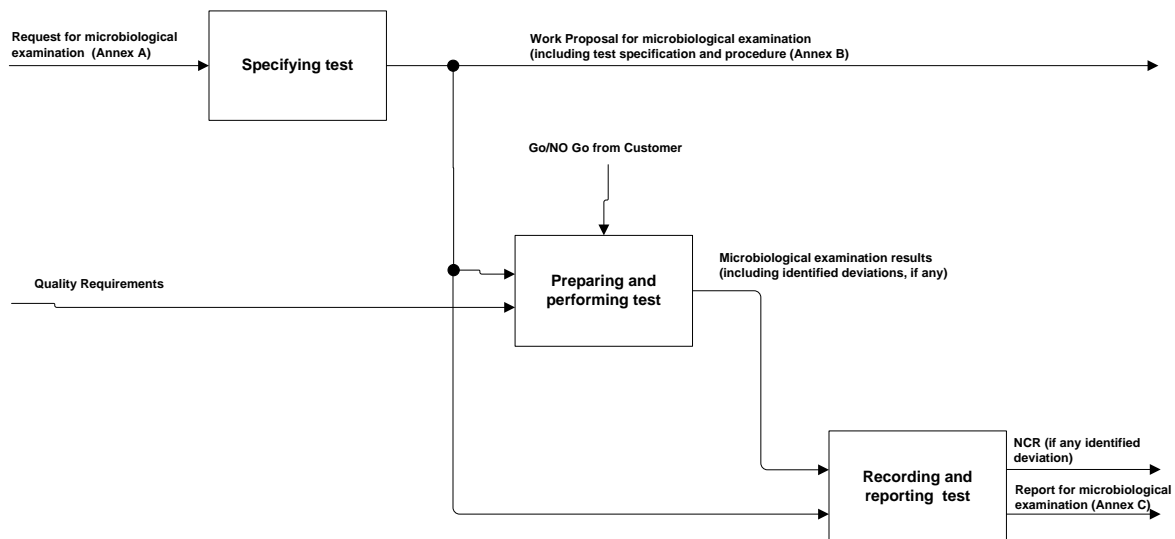


Figure 1 Microbiological examination process overview

Clause 5.1 provides the specification for the means of tests

Clause 5.2 and 5.3 provide the requirements for preparing, performing, recording and reporting microbiological examination.

5. Requirements

5.1. Specifying test

5.1.1. General provision

- a. The customer shall provide a request for microbiological examination according to Annex A DRD.
- b. ECSS-Q-20B shall be made applicable in the request for microbiological examination
- c. ECSS-Q-20-09B shall be made applicable in the request for microbiological examination
- d. For safety (e.g. hazard, health) and security (e.g. access control), the test centre shall comply with ECSS-Q-20-07A, clause 9.
- e. The supplier shall provide a microbiological examination proposal according to Annex B DRD.

5.1.2. Specifying the test means

5.1.2.1. Facilities

- a. The work area shall comply to the rules and guidelines of good laboratory practice.
- b. The ambient conditions for the process and work areas shall be $(22 \pm 3)^{\circ}\text{C}$ with a relative humidity of $(55 \pm 10)\%$ unless otherwise stated.

5.1.2.2. Equipment, reagents and consumables

The supplier shall identify and specify the list of the equipment, reagents and consumables necessary to set up and run the approved test procedures.

NOTE If the test procedures proposed in Annex D - Annex G are executed by the supplier, the corresponding equipment, reagents and consumables specification is described therein.

5.1.3. Specifying the test procedure

5.1.3.1. Test procedures

- a. A single swab shall be used for a surface area $\leq 25\text{ cm}^2$.
- b. Surface areas $\leq 1\text{ m}^2$ shall be samples with multiple swabs
- c. Surface areas $> 1\text{ m}^2$ shall be samples with wipes.
- d. Bioburden shall be determined with assays for quantification of aerobic mesophilic bacterial spores (and bacteria) that survive pasteurisation (heat shock at 80°C for 15 min).

NOTE 1 Example procedures are given in Annex D.1 (standard swab assay) and Annex E.1 (standard wipe assay)

NOTE 2 This sampling is only appropriate for materials that can tolerate sample collection using damp materials.

- e. Biodiversity shall be determined with assays for determination of the type of micro-organism.

NOTE 1 Example procedures are given in Annexes D.2 - D.5 (swabs) and Annexes E.2 - E.5 (wipes).

NOTE 2 In addition, non-culture-based methods are used for the molecular analysis of non-cultivable microorganisms with example procedures in Annex D.6 (swab) and Annex E.6.1 (wipe).

NOTE 3 This sampling is only appropriate for materials that can tolerate sample collection using damp materials.

NOTE 4 For cleanroom control also contact plates and active air sampler can be applied with example procedures in Annex F (contact plates) and Annex G (active air sampling).

NOTE 5 Multiple swabs and wipes may be used to sample a larger surface area.

- f. Air sampling shall be applied for continuous clean room monitoring.

5.2. Preparing and performing the microbiological examination

- a. The customer shall approve the microbiological examination proposal
- b. The test proposal shall include the procedures.
- c. ECSS-Q-20B shall apply for the establishment of the test procedures.

5.2.1. Preparing microbiological assays

5.2.1.1. Identification

- a. Locations where microbiological assays are taken shall be clearly identified with appropriate details to maintain traceability (e.g. microbiological assay plan).
- b. Assays shall be identified as a minimum by:
 1. Date and time
 2. Operator(s)
 3. Reference to trace location of assay
 4. Type of assay
 5. Area of the assay or air volume taken
 6. Storage conditions of assay sample

5.2.1.2. Preparation, handling and storage of reagents and consumables

Rules and guidelines of good laboratory practice shall be followed.

5.2.2. Performing microbiological assays

The supplier shall run the approved microbiological examination procedures as described in Annex B –DRD.

NOTE Examples of approved microbiological assay procedures are given in Annex D - Annex G.

5.3. Recording and reporting the test results

5.3.1. Test records

- a. The test records of the microbiological examination shall be retained for, at least, ten years or in accordance with project contract requirements.
- b. The quality records shall contain as a minimum the following:
 1. Date and time of the assay campaign
 2. Operator(s)
 3. Type of assay and assay procedure
 4. Traceable details about assay (e.g. location, area, air volume)
 5. Storage conditions of assay sample (e.g. temperature after sampling at packaging, transport time, temperature at start of assay)
 6. Details of the equipment used for the assay
 7. Comments concerning any unusual occurrence during sampling, transport or assay
 8. Reports of the microbiological examination results
- c. The test records of the microbiological examination shall be composed of:
 1. the request for microbiological examination according to Annex A –DRD,
 2. the microbiological examination proposal according to Annex B – DRD,
 3. the microbiological examination report according to Annex C – DRD,
 4. a conclusion with respect to the compliance with the project requirements (acceptance criteria) and associated non-conformances.

5.3.2. Test report

- a. The supplier shall apply ECSS-Q-20B, subclause 9.3.2 for the establishment of the test report.
- b. The supplier shall submit the test report to the customer for approval

5.3.3. Acceptance criteria and non-conformance

- a. Acceptance criteria shall be defined (beforehand) in common agreement between the test authority and the customer.
- b. Any suspected or actual equipment failure shall be recorded as project non conformance report so that previous results may be examined to ascertain whether or not re-inspection and re-testing.
- c. The test procedures shall contain an instantiation or adaptation for the test item of the non conformance processing flow chart as described in ECSS Q 20 09B.

NOTE In the frame of research and development activities, this is not necessary.

- d. The supplier shall notify the customer of the non conformance details.
- e. Traceability shall be maintained throughout the process from incoming inspection to final measurements and calculations, including details of the test equipment and personnel employed in performing the task.
- f. Evaluation of other properties may be invoked by the customer

Annex A (normative)

Request for microbiological examination - DRD

A.1. DRD identification

A.1.1. Requirement identification and source document

ECSS-Q-70-55, requirement.

A.1.2. Purpose and objective

The purpose of the request is the quantitative and/or qualitative microbiological examination of surfaces from flight hardware, cleanrooms, and microbiological examination of cleanroom air.

A.2. Expected response

A.2.1. Identification

The requirements for documentation management and control in ECSS-M-50B shall apply.

A.2.2. Scope and Content

The Request for microbiological examination shall include or refer to the following information

- a. objective of the test activity,
- b. background and justification to the test activity,
- c. flight hardware or cleanroom facilities to be investigated (including state of controlled environment at rest and operation),
- d. description of test activity, and
- e. deliverables.

Annex B (normative)

Microbiological examination test specifications and procedures (Work Proposal) - DRD

B.1. DRD identification

B.1.1. Requirement identification and source document

ECSS-Q-70-55, requirement.

B.1.2. Purpose and objective

The work proposal is a document that specifies the test activity for the microbiological examination. It is proposed by the test house. The work proposal for microbiological examination is prepared by the supplier who is responsible for the test activity, and it is submitted to the customer for review and approval.

B.2. Expected response

B.2.1. Identification

The requirements for documentation management and control in ECSS-M-50B shall apply.

B.2.2. Scope and content

The WP shall include or refer to the following information:

- a. A proposed work description giving:
 - 1. the objectives of the test activity,
 - 2. test procedure and reference to standards (this includes sources,),
 - 3. identification of flight hardware or cleanroom facilities (including state of controlled environment at rest and operation),
 - 4. test conditions (i.e. environment, properties evaluated and measurement techniques),
 - 5. expected test output.
- g. A proposed settlement describing the test procedures and any deviation from the conditions initially requested by the customer
- h. A financial and administrative proposal including:
 - 1. responsible person for the activity,
 - 2. list of deliverable items,
 - 3. work breakdown structure defining the required operations (i.e. preparation of specimens, testing, evaluation of results, reporting) and responsibilities,

4. time schedule,
5. itemized cost list.

Annex C (normative)

Microbiological examination test report - DRD

C.1. DRD identification

C.1.1. Requirement identification and source document

ECSS-Q-70-55, requirement.

C.1.2. Purpose and objective

The purpose of the microbiological examination test report is to provide quantitative and/or qualitative evidence of bioburden on sampled surfaces or cleanroom air.

C.2. Expected response

C.2.1. Identification

The requirements for documentation management and control in ECSS-M-50B shall apply.

C.2.2. Scope and content

a. The microbiological examination test report shall include or refer to the following information:

1. description of the purpose, objective, content and the reason prompting its preparation,
2. description of the sampled flight hardware or cleanroom or a reference to the document containing its identification characteristics (e.g. request for microbiological examination DRD),
3. calibration tools,
4. the microbiological assay procedures or a reference to the document containing the description of the test procedure (e.g. microbiological assay specifications and procedures DRD),

NOTE It often consist in describing the as- run test procedure as well as any deviation from the initial test procedure (including a discussion of possible effect on test).

5. the test results,
6. statistical analysis,
7. discussion about the test results,
8. conclusion and recommendations.

Annex D (informative) Procedures for swab assays

D.1. Swab assay 1 (standard swab assay)

D.1.1. General

The flow-chart for the swab assay 1 is schematically shown in Figure D-1.

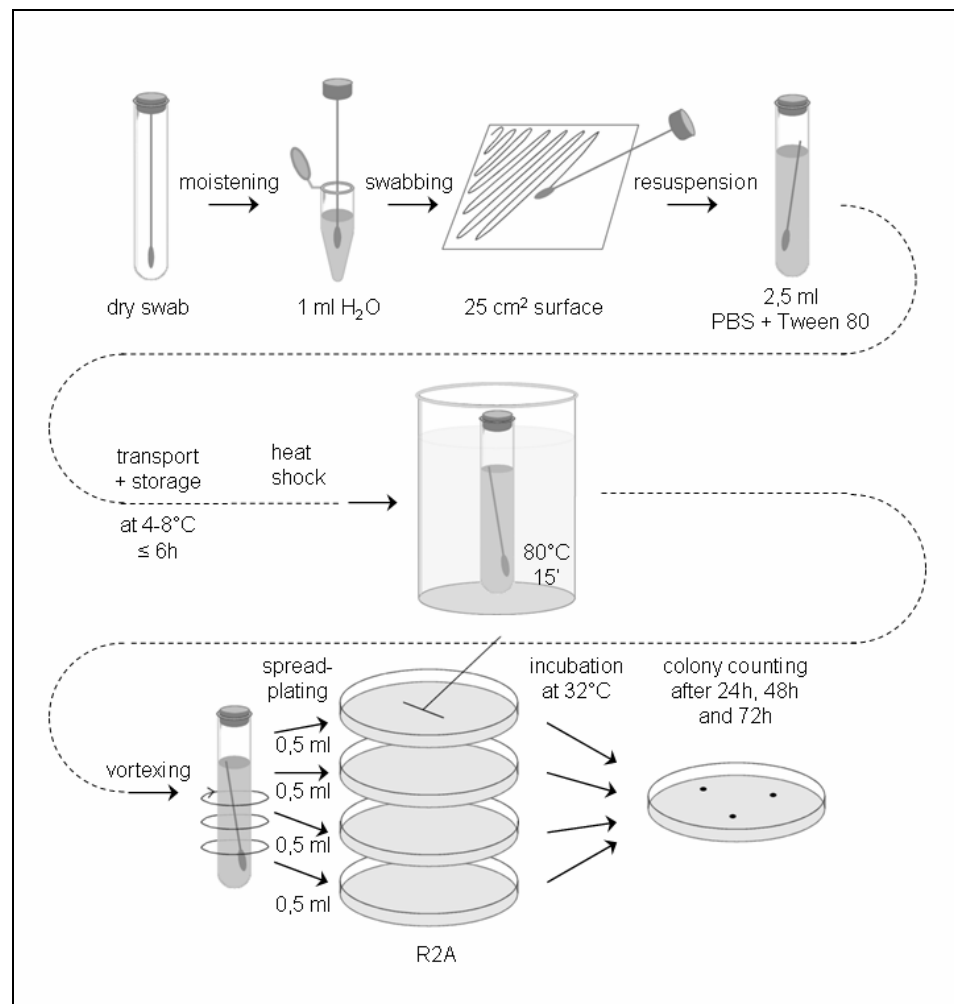


Figure D-1 : Flow chart for the standard swab assay (swab assay 1)

D.1.2. Sample collection

Prepare a sufficient number of sterile swabs and test tubes with sterile water, ASTM type IIB, for all swab samples to be collected, plus controls. Only Nylon flocked swabs shall be used. Aseptically remove a sterile swab from its container and moisten the head of the swab in a test tube with sterile water, ASTM type IIB,. Express excess moisture from the swab against the interior wall of the tube.

Hold the swab so that the handle makes about a 30° angle with the surface to be sampled. While moving the swab in one direction, rotate the head of the swab slowly and thoroughly over a measured 25 cm² surface area. Change the linear direction of the swabbing motion 90° and again swab the surface thoroughly. Complete a third coverage of the surface by again changing the direction of the swabbing motion by 135°.

Return the swab head to a tube containing 2.5 ml sterile buffer (PBS + 0.02 v/v % Tween 80, pH 7.2) by breaking the swab shaft at the breakpoint.

D.1.3. Transport and storage

Transport samples to the laboratory and store at 4 - 8°C and process within 24 hours.

D.1.4. Extraction

Place each tube containing the buffer and the swab on a vortex mixer and vortex at maximum power for 5 - 6 seconds.

D.1.5. Heat shock

Place the tube containing the vortexed suspension and the swab in a water bath at (80 ± 2) °C for 15 minutes, as determined by a pilot tube containing a thermometer. Make certain the water bath level is above the level of the liquid content of each tube being heated.

After heat shock, cool the tubes rapidly to bring the contents to $(30 - 35)$ °C. If the entire plating procedure requires more than 10 minutes, the heat shocked tubes shall be placed in an ice bath for no longer than 45 minutes prior to plating.

D.1.6. Plating

Vortex swab extraction suspension for 5 – 6 s and aseptically pipette 0.5 ml aliquots of the swab extraction suspension onto the surface of R2A Petri plates, using 2 ml total.

Use a sterile spreader to spread the dilution over the surface as evenly as possible. Allow the moisture to be absorbed into the agar before incubation.

D.1.7. Incubation

Plates shall be incubated inverted at (32 ± 1) °C.

D.1.8. Counting

Examine the sample plates at 24 and 48 hours. If colonies visible by eyes are observed, count and record data. Examine and record final colony counts at 72 hours. Do not remove the Petri plate covers until the final 72 hour count is made.

D.1.9. Controls

For each ten or fewer samples collected, also collect a 'field negative' control, at least 3 per day. Remove the sterile swab from its container, moisten with sterile water, ASTM type IIB, as above, wave the swab through the air for 2 to 4 seconds, and return the swab to a tube containing sterile buffer.

In the lab, create at least two 'lab negative controls' by moistening the head of a sterile swab in sterile water, as above, and return the swab to the tube containing sterile grade 3 buffer without exposing it to air.

Analyse the controls in the same way as the samples described above.

D.1.10. Equipment, reagents and consumable materials

- Dry sterile swabs
- Tubes with sterile water, ASTM type IIB, for moistening of swabs
- Tubes with 2.5 ml sterile buffer (PBS + 0.02 v/v % Tween 80, pH 7.2)
- Refrigerator (4 – 8)°C
- Vortex mixer
- Water bath (80 ± 2)°C
- Ice bath
- Thermometer
- Microliter pipette and sterile tips
- Laminar flow hood
- Sterile spreaders
- R2A agar plates (90 mm)
- Incubator (32 ± 1)°C

D.2. Swab assay 2

D.2.1. General

With this assay aerobic mesophiles are determined. The flow-chart for the swab assay 1 is schematically shown in Figure D-2.

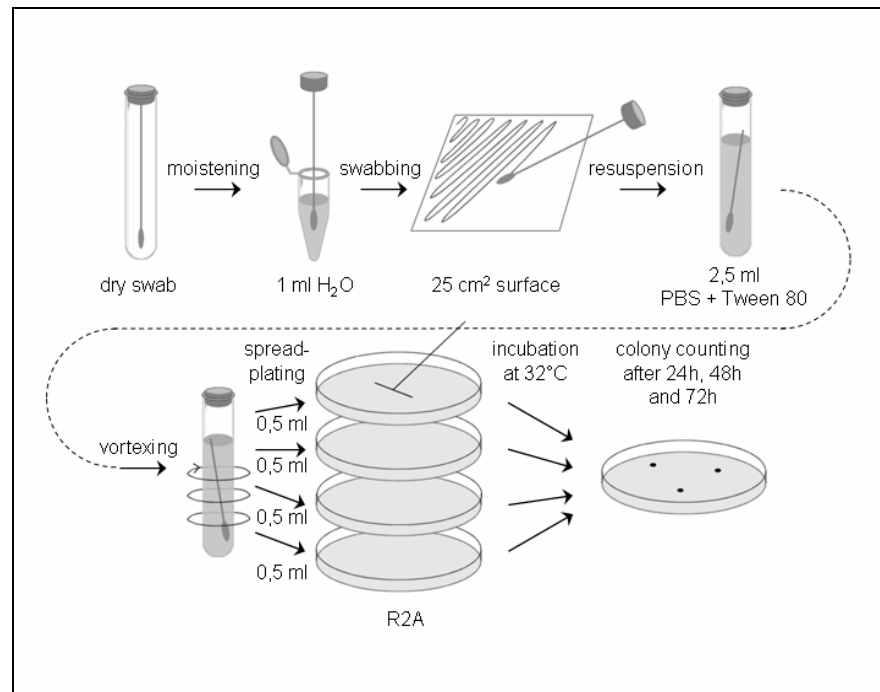


Figure D-2 : Flow chart for the standard swab assay (swab assay 1)

D.2.2. Sample collection

Prepare a sufficient number of sterile swabs and test tubes with sterile water, ASTM type IIB, for all swab samples to be collected, plus controls. Only Nylon flocked swabs shall be used. Aseptically remove a sterile swab from its container and moisten the head of the swab in a test tube with sterile water, ASTM type IIB,. Express excess moisture from the swab against the interior wall of the tube.

Hold the swab so that the handle makes about a 30° angle with the surface to be sampled. While moving the swab in one direction, rotate the head of the swab slowly and thoroughly over a measured 25 cm² surface area. Change the linear direction of the swabbing motion 90° and again swab the surface thoroughly. Complete a third coverage of the surface by again changing the direction of the swabbing motion by 135°.

Return the swab head to a tube containing 2.5 ml sterile buffer (PBS + 0.02 v/v % Tween 80, pH 7.2) by breaking the swab shaft at the breakpoint.

D.2.3. Transport and storage

Transport samples to the laboratory and store at 4 - 8°C and process within 24 hours.

D.2.4. Extraction

Place each tube containing the buffer and the swab on a vortex mixer and vortex at maximum power for 5 - 6 seconds.

NOTE Do not perform a heat-shock!

D.2.5. Plating

Vortex swab extraction suspension for 5 – 6 s and aseptically pipette 0.5 ml aliquots of the swab extraction suspension onto the surface of R2A Petri plates, using 2 ml total.

Use a sterile spreader to spread the dilution over the surface as evenly as possible. Allow the moisture to be absorbed into the agar before incubation.

D.2.6. Incubation

Plates shall be incubated inverted at $(32 \pm 1)^\circ\text{C}$.

D.2.7. Counting

Examine the sample plates at 24 and 48 hours. If colonies visible by eyes are observed, count and record data. Examine and record final colony counts at 72 hours. Do not remove the Petri plate covers until the final 72 hour count is made. Keeping photographic evidence for biodiversity determination might be helpful. If necessary, replant the colonies before identification and archiving.

D.2.8. Controls

For each ten or fewer samples collected, also collect a 'field negative' control, at least 3 per day. Remove the sterile swab from its container, moisten with sterile water, ASTM type IIB, as above, wave the swab through the air for 2 to 4 seconds, and return the swab to a tube containing sterile buffer.

In the lab, create at least two 'lab negative controls' by moistening the head of a sterile swab in sterile water, as above, and return the swab to the tube containing sterile grade 3 buffer without exposing it to air.

Analyse the controls in the same way as the samples described above.

D.3. Swab assay 3

D.3.1. General

With this assay aerobic psychrophiles / psychrotolerants are determined.

D.3.2. Sample collection

Prepare a sufficient number of sterile swabs and test tubes with sterile water, ASTM type IIB, for all swab samples to be collected, plus controls. Only Nylon flocked swabs shall be used. Aseptically remove a sterile swab from its container and moisten the head of the swab in a test tube with sterile water, ASTM type IIB,. Express excess moisture from the swab against the interior wall of the tube.

Hold the swab so that the handle makes about a 30° angle with the surface to be sampled. While moving the swab in one direction, rotate the head of the swab slowly and thoroughly over a measured 25 cm² surface area. Change the linear direction of the swabbing motion 90° and again swab the surface thoroughly. Complete a third coverage of the surface by again changing the direction of the swabbing motion by 135°.

Return the swab head to a tube containing 2.5 ml sterile buffer (PBS + 0.02 v/v % Tween 80, pH 7.2) by breaking the swab shaft at the breakpoint.

D.3.3. Transport and storage

Transport samples to the laboratory and store at 4 - 8°C and process within 24 hours.

D.3.4. Extraction

Place each tube containing the buffer and the swab on a vortex mixer and vortex at maximum power for 5 - 6 seconds.

NOTE Do not perform a heat-shock!

D.3.5. Plating

Vortex swab extraction suspension for 5 – 6 s and aseptically pipette 0.5 ml aliquots of the swab extraction suspension onto the surface of R2A Petri plates, using 2 ml total.

Use a sterile spreader to spread the dilution over the surface as evenly as possible. Allow the moisture to be absorbed into the agar before incubation.

D.3.6. Incubation

Two plates shall be incubated inverted at (10 ± 1)°C, the other two at (4 ± 1)°C.

D.3.7. Counting

Examine the sample plates at 14 and 21 days. If colonies visible by eyes are observed, count and record data. Examine and record final colony counts at 28 days. Do not remove the Petri plate covers until the final 28 days count is made. Keeping photographic evidence for biodiversity determination might be helpful. If necessary, replat the colonies before identification and archiving.

D.3.8. Controls

For each ten or fewer samples collected, also collect a 'field negative' control, at least 3 per day. Remove the sterile swab from its container, moisten with sterile water, ASTM type IIB, as above, wave the swab through the air for 2 to 4 seconds, and return the swab to a tube containing sterile buffer.

In the lab, create at least two 'lab negative controls' by moistening the head of a sterile swab in sterile water, as above, and return the swab to the tube containing sterile grade 3 buffer without exposing it to air.

Analyse the controls in the same way as the samples described above.

D.4. Swab assay 4

D.4.1. General

With this assay aerobic mesophilic fungi, that is yeasts and molds, are determined.

D.4.2. Sample collection

Prepare a sufficient number of sterile swabs and test tubes with sterile water, ASTM type IIB, for all swab samples to be collected, plus controls. Only Nylon flocked swabs shall be used. Aseptically remove a sterile swab from its container and moisten the head of the swab in a test tube with sterile water, ASTM type IIB,. Express excess moisture from the swab against the interior wall of the tube.

Hold the swab so that the handle makes about a 30° angle with the surface to be sampled. While moving the swab in one direction, rotate the head of the swab slowly and thoroughly over a measured 25 cm² surface area. Change the linear direction of the swabbing motion 90° and again swab the surface thoroughly. Complete a third coverage of the surface by again changing the direction of the swabbing motion by 135°.

Return the swab head to a tube containing 2.5 ml sterile buffer (PBS + 0.02 v/v % Tween 80, pH 7.2) by breaking the swab shaft at the breakpoint.

D.4.3. Transport and storage

Transport samples to the laboratory and store at (4 – 8)°C and process within 24 hours.

D.4.4. Extraction

Place each tube containing the buffer and the swab on a vortex mixer and vortex at maximum power for 5 - 6 seconds.

NOTE Do not perform a heat-shock.

D.4.5. Plating

Vortex swab extraction suspension for 5 – 6 s and aseptically pipette 0.5 ml aliquots of the swab extraction suspension onto the surface of PDA agar plates, using 2 ml total.

Use a sterile spreader to spread the dilution over the surface as evenly as possible. Allow the moisture to be absorbed into the agar before incubation.

D.4.6. Incubation

Plates shall be incubated inverted at (25 ± 1)°C.

D.4.7. Counting

Examine the sample plates at 3, 5 and 7 days. If colonies visible by eyes are observed, count and record data. If necessary, replate the colonies before identification and archiving. Keeping photographic evidence for biodiversity determination might be helpful.

D.4.8. Controls

For each ten or fewer samples collected, also collect a 'field negative' control, at least 3 per day. Remove the sterile swab from its container, moisten with sterile water, ASTM type IIB, as

above, wave the swab through the air for 2 to 4 seconds, and return the swab to a tube containing sterile buffer.

In the lab, create at least two 'lab negative controls' by moistening the head of a sterile swab in sterile water, as above, and return the swab to the tube containing sterile grade 3 buffer without exposing it to air.

Analyse the controls in the same way as the samples described above.

D.5. Swab assay 5

D.5.1. General

With this assay anaerobic mesophiles are determined.

D.5.2. Sample collection

Before sampling, prepare a sufficient number of tubes with anaerobic buffer (PBS + 0.02 v/v % Tween 80). Add sodium resazurin (0.001 g/l) as redox indicator. Reduce PBS by adding 0.5 g/l sodium sulfide x 9 H₂O. Adjust pH to 7.0. Under anaerobic conditions, prepare 10 ml aliquots in glass tubes closed with butyl rubber stoppers and clamps under anaerobic conditions. Add nitrogen gas phase. Autoklave.

Prepare a sufficient number of sterile swabs and test tubes with sterile water, ASTM type IIB, for all swab samples to be collected, plus controls. Only Nylon flocked swabs shall be used.

Directly before sampling, remove clamp of a tube with anaerobic buffer.

Aseptically remove a sterile swab from its container and moisten the head of the swab in a test tube with sterile water, ASTM type IIB. Express excess moisture from the swab against the interior wall of the tube.

Hold the swab so that the handle makes about a 30° angle with the surface to be sampled. While moving the swab in one direction, rotate the head of the swab slowly and thoroughly over a measured 25 cm² surface area. Change the linear direction of the swabbing motion 90° and again swab the surface thoroughly. Complete a third coverage of the surface by again changing the direction of the swabbing motion by 135°.

Open anaerobic tube, drop swab into PBS by breaking the swab shaft at the breakpoint, close tube immediately with a new sterile stopper. If the redox indicator turns red, reduce tube content by adding 0.1 ml of a mixture of cystein-HCl and sodium sulfide by using a sterile disposable syringe and a hypodermic needle. Increase the concentration of reducing agents stepwise (0.1 ml), if necessary.

D.5.3. Transport and storage

Transport samples to the laboratory and store at (4 – 8)°C and process within 24 hours.

D.5.4. Extraction

Place each tube containing the buffer and the swab on a vortex mixer and vortex at maximum power for 5 - 6 seconds.

NOTE Do not perform a heat-shock.

D.5.5. Plating

Perform plating under anaerobic conditions. Vortex swab extraction suspension for 5 – 6 s and aseptically pipette 0.5 ml aliquots of the swab extraction suspension onto the surface of Thioglykollate agar plates, using 2 ml total.

Use a sterile spreader to spread the dilution over the surface as evenly as possible. Allow the moisture to be absorbed into the agar before incubation.

D.5.6. Incubation

Plates shall be incubated inverted at (32 ± 1)°C under anaerobic conditions.

D.5.7. Counting

Examine the sample plates at 3 days, 7 days and 14 days. If colonies visible by eyes are observed, count and record data. If necessary, replate the colonies under anaerobic conditions before identification and archiving. Keeping photographic evidence for biodiversity determination might be helpful.

D.5.8. Controls

For each ten or fewer samples collected, also collect a 'field negative' control, at least 3 per day. Remove the sterile swab from its container, moisten with sterile water, ASTM type IIB, as above, wave the swab through the air for 2 to 4 seconds, and return the swab to a tube containing sterile buffer.

In the lab, create at least two 'lab negative controls' by moistening the head of a sterile swab in sterile water, as above, and return the swab to the tube containing sterile grade 3 buffer without exposing it to air.

Analyse the controls in the same way as the samples described above.

D.6. Swab assay 6

D.6.1. General

With this assay DNA is isolated from samples, the 16S rDNA is amplified, cloned and sequenced.

NOTE Methods for taxonomic allocation deduced by sequence comparison with public 16S rDNA data bases are not part of this standard.

D.6.2. Sample collection

Prepare a sufficient number of sterile swabs and test tubes with sterile DNase- and RNase-free ultrapure water for all swab samples to be collected, plus controls. Only Nylon flocked swabs shall be used. Aseptically remove a sterile swab from its container and moisten the head of the swab in a test tube with sterile DNase- and RNase-free ultrapure water. Express excess moisture from the swab against the interior wall of the tube.

Hold the swab so that the handle makes about a 30° angle with the surface to be sampled. While moving the swab in one direction, rotate the head of the swab slowly and thoroughly over a measured 25 cm² surface area. Change the linear direction of the swabbing motion 90° and again swab the surface thoroughly. Complete a third coverage of the surface by again changing the direction of the swabbing motion by 135°.

Return the swab to a tube containing 2.0 ml sterile DNase- and RNase-free ultrapure water by breaking the swab shaft at the breakpoint.

D.6.3. Transport and storage

Transport samples to the laboratory and store at (4 – 8)°C and process within 24 hours.

D.6.4. Extraction

Add 2 ml of 2x suspending buffer to each sample. Add small amount of lysozyme (covering the tip of a spatula) and incubate at 37 °C for 30 min. Add 0.2 ml SDS (final concentration 1 %) and add Proteinase K (200 µg/ml), incubate at 65 °C for 30 min. Freeze and thaw sample twice. Add 4 ml of phenol, mix suspension gently. Centrifuge at 15 °C for 5 min with 8,000 rpm. Carefully decant or pipette aqueous layer into a new tube. Add 4 ml of a 1:1 mixture of phenol:chloroform-isoamylalcohol (24:1), mix gently, centrifuge at 15 °C for 5 min with 8,000 rpm. Transfer aqueous layer into a new tube. Repeat last step with chloroform-isoamylalcohol (24:1). Transfer aqueous layer into a new tube, precipitate DNA with 0.6 volumes isopropanol. Alternatively use 2 volumes ethanol and 20 mg/ml glycogen. Incubate at -20 °C overnight. Transfer suspension into small tubes for centrifugation at 13000 rpm at 4°C for 30 min. Wash pellet with 70 % ethanol (ice cold). Repeat washing step. Dry pellet. Solve pellet in TE buffer or water.

D.6.5. DNA amplification by PCR

The following to be used for amplification of 16S rDNA from Archaea and Bacteria are shown in Table D-1.

Target	Forward (5' – 3')	Reverse (5' – 3')	Reference
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Archaea	345af CGGGGYGCASCAGGCGCGAA	1406uR ACGGGCGGTGTGTRCAA	Burggraf et al., 1997; Lane, 1991
Bacteria	9bf GRGTTTGATCCTGGCTCAG	1406uR ACGGGCGGTGTGTRCAA	Burggraf et al., 1992; Lane, 1991

Table D-1 : Primers for amplification of 16S rDNA from Archaea and Bacteria

D.6.6. PCR conditions

The following notes shall be taken into account for the PCR conditions

- NOTE 1 Usage of LowDNA-Taq or Pfu Taq for bacterial PCR is recommended.
- NOTE 2 The preparation of the PCR mixtures under sterile conditions (sterile hood), using aerosol filter pipette tips, is strongly recommended for environmental samples.
- NOTE 3 For each primer combination a negative control (water instead of DNA) and a positive control is recommended.
- NOTE 4 Hotstart- PCR is recommended.

The following composition shall be used for a 25 µl PCR assay:

- PCR buffer, incl. MgCl₂ (10x) 2.5 µl
- Nucleotide mix (10 mM each) 0.5 µl
- F-Primer (25 ng/µl) 1.0 µl
- R-Primer (25 ng/µl) 1.0 µl
- (LD)-Taq (5 U/µl) 0.125 µl
- Template DNA (10 ng/µl) 1.0 µl
- H₂O, sterile, DNA free Ad 25 µl

The PCR cycles are shown in Table D-2

Cycles	Time, s	Temperature, °C
1	90	96
10	30	96
	30	55
	60	72
25-30	20	94
	30	55
	60 ^a	72
1	600	72
1	> 600	4

^a +2 in each cycle

Table D-2 : PCR cycles

After PCR, the length of the PCR product obtained shall be checked on a agarose gel. The PCR product can now be used for sequencing reactions or cloning for environmental samples.

D.6.7. Cloning of PCR products

Cloning shall be done according the manufacturer's recommendations, as given in the kit manual.

D.6.8. Screening of clones

The presence of inserts of the expected sizes shall be analyzed by direct PCR screening of transformants without plasmid extraction.

Pick a small part of each colony, using tooth picks (sterile) and perform colony PCR with the plasmid-specific primers (streak the same tooth pick on a new agar plate for storage of the colony). Check the sizes of the inserts by electrophoresis (use 10 μ l of PCR product). Subject the remaining PCR product to RFLP analysis, using the enzymes AluI, HhaI, HinfI and RsaI according manufacturer's recommendations. Select representative transformants on the basis of the 16S rRNA gene fingerprint pattern. Grow these in liquid medium and purify plasmids for sequencing reaction.

D.6.9. Controls

For each step of the method, appropriate controls are recommended. For DNA extraction, a field blank control, as well as a lab control and a water control shall be performed.

Field blank controls

Expose wipe or swab to the air in the clean room during sampling (wave) and proceed with this control like with the samples

Lab control

Expose wipe or swab to the air in the lab laminar flow and proceed with this control like with the samples.

Water control

Instead of swab sample extract use sterile DNase- and RNase-free ultrapure water for DNA extraction.

For PCR use the three blanks mentioned above. Additionally use one PCR blank (water instead of sample). For PCR the usage of positive controls is also strongly recommended.

Analyse the controls in the same way as the samples described above.

Annex E (informative)

Procedures for wipe assays

E.1. Wipe assay 1 (standard wipe assay)

E.1.1. General

The wipe assay corresponds basically to the swab assay procedure described in Annex D.1.

E.1.2. Sample collection

Prepare a sufficient number of sterile 15 cm x 15 cm wipes prewetted with 3 ml water, ASTM type IIB, and sterile transport tubes or jars with buffer (20 ml PBS + 0.02 v/v % Tween 80, pH 7.2) to accommodate all samples to be collected, plus controls.

Rinse gloves with 70% isopropyl alcohol between each sample, and change gloves at least once every 4 samples.

Place the wipe flat on the sample surface and rub over the entire surface using a firm, steady pressure. Refold the wipe by reversing the direction of the open fold so the contaminated surface is interior in the new configuration. Rub the wipe over the sample area a total of three times, rotating the direction of motion 90 degrees and 135 degrees, respectively, after each complete sampling of the area. Transfer the wipe into a sterile transport jar or tube.

E.1.3. Transport and storage

Transport samples to the laboratory and store at (4 – 8)°C and process within 24 hours.

E.1.4. Extraction

Add 20 ml of sterile buffer (PBS + 0.02 v/v % Tween 80, pH 7.2) to each sample and reseal the jar or tube.

Vortex at maximum speed for 5-10 seconds, even if not very efficient for jars. Alternatively, if the wipe is in a jar that can be sealed tightly, close the lid and shake vigorously for 15 seconds.

Suspend sample jars or tubes in an ultrasonic bath, making sure the liquid level in the bath is above the fluid level in the sample jars or tubes and that the number of jars or tubes does not exceed the performance rating of the sonicator. Sonicate for (120 ± 5) s.

E.1.5. Heat shock

Place the jar or tube containing the vortexed and sonicated suspension and the wipe in a water bath at (80 ± 2)°C for 15 minutes, as determined by a pilot jar or tube containing a thermometer. Make certain the water bath level is above the level of the liquid content of each jar or tube being heated.

After heat shock, cool the jars or tubes rapidly to bring the contents to 30 – 35 °C. If the entire plating procedure requires more than 10 minutes, the heat shocked jars or tubes shall be placed in an ice bath for no longer than 45 minutes prior to plating.

E.1.6. Plating

If necessary, make appropriate dilutions of the wipe extraction suspension in sterile buffer (PBS + 0.02 v/v % Tween 80, pH 7.2).

Vortex wipe extraction suspension for 5 – 6 s and aseptically pipette 4.0 ml portions of the suspension into individual sterile 90 mm Petri plates. A total of 32 ml of suspension shall be plated.

Add 20 ml sterile, molten (48 – 50)°C R2A to each plate and mix the contents by gentle swirling, and allow the mixture to solidify at room temperature.

E.1.7. Incubation

Plates shall be incubated inverted at (32 ± 1)°C.

E.1.8. Counting

Examine the sample plates at 24 and 48 hours. If colonies visible by eyes are observed, count and record data. Examine and record final colony counts at 72 hours. Do not remove the Petri plate covers until the final 72 hour count is made.

E.1.9. Controls

For each six or fewer samples collected, also collect one 'field negative' control.

Remove a sterile 15 cm x 15 cm wipe prewetted with 3 ml water, ASTM type IIB, from its transport tube, wave the wipe through the air for approximately 2 to 4 seconds, and place the wipe in a sterile transport tube or jar with buffer (20 ml PBS + 0.02 v/v % Tween 80, pH 7.2).

In the lab, create at least two 'lab negative controls' by placing a sterile 15 cm x 15 cm wipe prewetted with 3 ml water, ASTM type IIB, and insert it into in a sterile transport tube or jar with buffer (20 ml PBS + 0.02 v/v % Tween 80, pH 7.2) without exposing it to air.

Analyse the controls in the same way as the samples described above.

E.1.10. Equipment, reagents and consumable materials

Sterile 15 cm x 15 cm wipes prewetted with 3 ml water, ASTM type IIB,

Transport tubes or jars with sterile buffer (20 ml PBS + 0.02 v/v % Tween 80, pH 7.2)

Refrigerator (4 – 8)°C

Vortex mixer

Water bath (80 ± 2) °C

Ice bath

Thermometer

Pipette and tips

Laminar flow hood

Sterile spreaders

R2A agar plates (90 mm)

Incubator (32 ± 1)°C

Sterile gloves

70 % IPA

E.2. Wipe assay 2

E.2.1. General

With this assay aerobic mesophiles are determined. The wipe assay corresponds basically to the swab assay procedures described in Annex D.2.

NOTE Standard membrane filter methods may be used to test extraction suspension, as an alternative to these procedures.

E.2.2. Sample collection

Prepare a sufficient number of sterile 15 cm x 15 cm wipes prewetted with 3 ml water, ASTM type IIB, and sterile transport tubes or jars with buffer (20 ml PBS + 0.02 v/v % Tween 80, pH 7.2) to accommodate all samples to be collected, plus controls.

Rinse gloves with 70% isopropyl alcohol between each sample, and change gloves at least once every 4 samples.

Place the wipe flat on the sample surface and rub over the entire surface using a firm, steady pressure. Refold the wipe by reversing the direction of the open fold so the contaminated surface is interior in the new configuration. Rub the wipe over the sample area a total of three times, rotating the direction of motion 90 degrees and 135 degrees, respectively, after each complete sampling of the area. Transfer the wipe into a sterile transport jar or tube.

E.2.3. Transport and storage

Transport samples to the laboratory and store at (4 – 8)°C and process within 24 hours.

E.2.4. Extraction

Add 20 ml of sterile buffer (PBS + 0.02 v/v % Tween 80, pH 7.2) to each sample and reseal the jar or tube.

Vortex at maximum speed for 5-10 seconds, even if not very efficient for jars. Alternatively, if the wipe is in a jar that can be sealed tightly, close the lid and shake vigorously for 15 seconds.

Suspend sample jars or tubes in an ultrasonic bath, making sure the liquid level in the bath is above the fluid level in the sample jars or tubes and that the number of jars or tubes does not exceed the performance rating of the sonicator. Sonicate for (120 ± 5) s.

NOTE Do not perform a heat-shock.

E.2.5. Plating

If necessary, make appropriate dilutions of the wipe extraction suspension in sterile buffer (PBS + 0.02 v/v % Tween 80, pH 7.2).

Vortex wipe extraction suspension for 5 – 6 s and aseptically pipette 4.0 ml portions of the suspension into individual sterile 90 mm Petri plates. A total of 32 ml of suspension shall be plated.

Add 20 ml sterile, molten (48 – 50)°C R2A to each plate and mix the contents by gentle swirling, and allow the mixture to solidify at room temperature.

E.2.6. Incubation

Plates shall be incubated inverted at (32 ± 1)°C.

E.2.7. Counting

Examine the sample plates at 24 and 48 hours. If colonies visible by eyes are observed, count and record data. Examine and record final colony counts at 72 hours. Do not remove the Petri plate covers until the final 72 hour count is made.

E.2.8. Controls

For each six or fewer samples collected, also collect one 'field negative' control.

Remove a sterile 15 cm x 15 cm wipe prewetted with 3 ml water, ASTM type IIB, from its transport tube, wave the wipe through the air for approximately 2 to 4 seconds, and place the wipe in a sterile transport tube or jar with buffer (20 ml PBS + 0.02 v/v % Tween 80, pH 7.2).

In the lab, create at least two 'lab negative controls' by placing a sterile 15 cm x 15 cm wipe prewetted with 3 ml water, ASTM type IIB, and insert it into in a sterile transport tube or jar with buffer (20 ml PBS + 0.02 v/v % Tween 80, pH 7.2) without exposing it to air.

Analyse the controls in the same way as the samples described above.

E.3. Wipe assay 3

E.3.1. General

With this assay aerobic psychrophile / psychrotolerants are determined. The wipe assay corresponds basically to the swab assay procedures described in Annex D.3.

NOTE Standard membrane filter methods may be used to test extraction suspension, as an alternative to these procedures.

E.3.2. Sample collection

Prepare a sufficient number of sterile 15 cm x 15 cm wipes prewetted with 3 ml water, ASTM type IIB, and sterile transport tubes or jars with buffer (20 ml PBS + 0.02 v/v % Tween 80, pH 7.2) to accommodate all samples to be collected, plus controls.

Rinse gloves with 70% isopropyl alcohol between each sample, and change gloves at least once every 4 samples.

Place the wipe flat on the sample surface and rub over the entire surface using a firm, steady pressure. Refold the wipe by reversing the direction of the open fold so the contaminated surface is interior in the new configuration. Rub the wipe over the sample area a total of three times, rotating the direction of motion 90 degrees and 135 degrees, respectively, after each complete sampling of the area. Transfer the wipe into a sterile transport jar or tube.

E.3.3. Transport and storage

Transport samples to the laboratory and store at (4 – 8)°C and process within 24 hours.

E.3.4. Extraction

Add 20 ml of sterile buffer (PBS + 0.02 v/v % Tween 80, pH 7.2) to each sample and reseal the jar or tube.

Vortex at maximum speed for 5-10 seconds, even if not very efficient for jars. Alternatively, if the wipe is in a jar that can be sealed tightly, close the lid and shake vigorously for 15 seconds.

Suspend sample jars or tubes in an ultrasonic bath, making sure the liquid level in the bath is above the fluid level in the sample jars or tubes and that the number of jars or tubes does not exceed the performance rating of the sonicator. Sonicate for (120 ± 5) s.

NOTE Do not perform a heat-shock.

E.3.5. Plating

If necessary, make appropriate dilutions of the wipe extraction suspension in sterile buffer (PBS + 0.02 v/v % Tween 80, pH 7.2).

Vortex wipe extraction suspension for (5 – 6) s and aseptically pipette 4.0 ml portions of the suspension into individual sterile 90 mm Petri plates. A total of 32 ml of suspension shall be plated.

Add 20 ml sterile, molten (48 – 50)°C R2A to each plate and mix the contents by gentle swirling, and allow the mixture to solidify at room temperature.

E.3.6. Incubation

Four plates shall be incubated inverted at $(10 \pm 1)^\circ\text{C}$, the other four at $(4 \pm 1)^\circ\text{C}$.

E.3.7. Counting

Examine the sample plates at 14 and 21 days. If colonies visible by eyes are observed, count and record data. Examine and record final colony counts at 28 days. Do not remove the Petri plate covers until the final 28 days count is made. Keeping photographic evidence for biodiversity determination might be helpful. If necessary, replate the colonies before identification and archiving.

E.3.8. Controls

For each six or fewer samples collected, also collect one 'field negative' control.

Remove a sterile 15 cm x 15 cm wipe prewetted with 3 ml water, ASTM type IIB, from its transport tube, wave the wipe through the air for approximately 2 to 4 seconds, and place the wipe in a sterile transport tube or jar with buffer (20 ml PBS + 0.02 v/v % Tween 80, pH 7.2).

In the lab, create at least two 'lab negative controls' by placing a sterile 15 cm x 15 cm wipe prewetted with 3 ml water, ASTM type IIB, and insert it into in a sterile transport tube or jar with buffer (20 ml PBS + 0.02 v/v % Tween 80, pH 7.2) without exposing it to air.

Analyse the controls in the same way as the samples described above.

E.4. Wipe assay 4

E.4.1. General

With this assay aerobic mesophilic fungi, that is yeasts and molds, are determined. The wipe assay corresponds basically to the swab assay procedures described in Annex D.4.

NOTE Standard membrane filter methods may be used to test extraction suspension, as an alternative to these procedures.

E.4.2. Sample collection

Prepare a sufficient number of sterile 15 cm x 15 cm wipes prewetted with 3 ml water, ASTM type IIB, and sterile transport tubes or jars with buffer (20 ml PBS + 0.02 v/v % Tween 80, pH 7.2) to accommodate all samples to be collected, plus controls.

Rinse gloves with 70% isopropyl alcohol between each sample, and change gloves at least once every 4 samples.

Place the wipe flat on the sample surface and rub over the entire surface using a firm, steady pressure. Refold the wipe by reversing the direction of the open fold so the contaminated surface is interior in the new configuration. Rub the wipe over the sample area a total of three times, rotating the direction of motion 90 degrees and 135 degrees, respectively, after each complete sampling of the area. Transfer the wipe into a sterile transport jar or tube.

E.4.3. Transport and storage

Transport samples to the laboratory and store at (4 – 8)°C and process within 24 hours.

E.4.4. Extraction

Add 20 ml of sterile buffer (PBS + 0.02 v/v % Tween 80, pH 7.2) to each sample and reseal the jar or tube.

Vortex at maximum speed for 5-10 seconds, even if not very efficient for jars. Alternatively, if the wipe is in a jar that can be sealed tightly, close the lid and shake vigorously for 15 seconds.

Suspend sample jars or tubes in an ultrasonic bath, making sure the liquid level in the bath is above the fluid level in the sample jars or tubes and that the number of jars or tubes does not exceed the performance rating of the sonicator. Sonicate for (120 ± 5)s.

NOTE Do not perform a heat-shock.

E.4.5. Plating

If necessary, make appropriate dilutions of the wipe extraction suspension in sterile buffer (PBS + 0.02 v/v % Tween 80, pH 7.2).

Vortex wipe extraction suspension for (5 – 6) s and aseptically pipette 4.0 ml portions of the suspension into individual sterile 90 mm Petri plates. A total of 32 ml of suspension shall be plated.

Add 20 ml sterile, molten (48 – 50)°C PDA to each plate and mix the contents by gentle swirling, and allow the mixture to solidify at room temperature.

E.4.6. Incubation

Plates shall be incubated inverted at (25 ± 1)°C.

E.4.7. Counting:

Examine the sample plates at 3, 5 and 7 days. If colonies visible by eyes are observed, count and record data. If necessary, replate the colonies before identification and archiving. Keeping photographic evidence for biodiversity determination might be helpful.

E.4.8. Controls

For each six or fewer samples collected, also collect one 'field negative' control.

Remove a sterile 15 cm x 15 cm wipe prewetted with 3 ml water, ASTM type IIB, from its transport tube, wave the wipe through the air for approximately 2 to 4 seconds, and place the wipe in a sterile transport tube or jar with buffer (20 ml PBS + 0.02 v/v % Tween 80, pH 7.2).

In the lab, create at least two 'lab negative controls' by placing a sterile 15 cm x 15 cm wipe prewetted with 3 ml water, ASTM type IIB, and insert it into in a sterile transport tube or jar with buffer (20 ml PBS + 0.02 v/v % Tween 80, pH 7.2) without exposing it to air.

Analyse the controls in the same way as the samples described above.

E.5. Wipe assay 5

E.5.1. General

With this assay anaerobic mesophiles are determined. The wipe assay corresponds basically to the swab assay procedures described in Annex D.5.

NOTE Standard membrane filter methods may be used to test extraction suspension, as an alternative to these procedures.

E.5.2. Sample collection

TBD

E.5.3. Transport and storage

Transport samples to the laboratory and store at $(4 - 8)^{\circ}\text{C}$ and process within 24 hours.

E.5.4. Extraction

TBD

Vortex at maximum speed for 5-10 seconds, even if not very efficient for jars. Alternatively, if the wipe is in a jar that can be sealed tightly, close the lid and shake vigorously for 15 seconds.

Suspend sample jars or tubes in an ultrasonic bath, making sure the liquid level in the bath is above the fluid level in the sample jars or tubes and that the number of jars or tubes does not exceed the performance rating of the sonicator. Sonicate for (120 ± 5) s.

NOTE Do not perform a heat-shock.

E.5.5. Plating

TBD

E.5.6. Incubation

TBD

E.5.7. Counting

TBD

E.5.8. Controls

TBD

Analyse the controls in the same way as the samples described above.

E.6. Wipe assay 6

E.6.1. General

With this assay DNA is isolated from samples, the 16S rDNA is amplified, cloned and sequenced.. The wipe assay corresponds basically to the swab assay procedures described in Annex D.6.

NOTE TBD

E.6.2. Sample collection

TBD

E.6.3. Transport and storage

TBD

E.6.4. Extraction

TBD

E.6.5. Plating

TBD

E.6.6. Incubation

TBD

E.6.7. Counting

TBD

E.6.8. Controls

TBD

Annex F (informative) Procedures for contact plates

F.1. Contact plates

F.1.1. General

With this assay aerobic mesophiles from plane, smooth and dry cleanroom or garments surfaces are determined.

F.1.2. Sample collection

An appropriate number of ready-to-use R2A contact plates of each purchased batch shall have been checked for certificate availability, visual integrity, sterility and fertility. The plates shall have been stored according to the manufacturer's recommendations and shall be within the storage conditions and period of validity insured by the manufacturer on the day of sampling until the end of the incubation period.

The circular growth area of commercially available contact plates is approximately 25 cm².

The sampling applicator, if used, shall be preliminary disinfected. At the sampling location, remove aseptically the contact plate lid and hold it side down, while holding with the other hand the applicator to be pressed on the targeted surface for 10 seconds without any lateral movement.

Alternatively, hold the plate with thumb and second finger and use the index finger to press the plate bottom firmly with a constant pressure against the surface during 10 seconds without any lateral movement.

Immediately upon sampling completion, close the contact plate with the lid and carefully wipe the surfaces with sterile disinfecting cleanroom wipes to remove any traces of residuals.

F.1.3. Transport and storage

Transport samples to the laboratory and store at (4 – 8) °C and process within 24 hours.

F.1.4. Incubation

Plates shall be incubated inverted at (32± 1)°C.

F.1.5. Counting

Examine the sample plates at 24 and 48 hours. If colonies visible by eyes are observed, count and record data. Examine and record final colony counts at 72 hours. Do not remove the Petri plate covers until the final 72 hour count is made.

F.1.6. Controls

For each ten or fewer samples collected, also collect a 'field negative' control, at least 3 per day obtained according to the procedure described above but without contacting the plate with the surfaces.

In the lab, create at least two 'lab negative controls' obtained according to the procedure described above but without contacting the plate with the surfaces.

Analyse the controls in the same way as the samples described above.

F.1.7. Equipment, reagents and consumable materials

- R2A contact plates
- Refrigerator (4 – 8)°C
- Laminar flow hood
- Incubator (32 ± 1)°C
- Sterile gloves

Annex G (informative)

Procedure for active air sampling

G.1. Wipe assay 1 (standard wipe assay)

G.1.1. General

An active air sampler is used to collect air samples using sterile gelatine membrane filters in order to determine the microbial contamination present in cleanrooms. With this assay aerobic mesophiles are determined.

G.1.2. Sample collection

An appropriate number of gelatine filters of each purchased batch shall have been checked for certificate availability, visual integrity, sterility and fertility on R2A plates. The filters shall have been stored according to the manufacturer's recommendations and shall be within the storage conditions and period of validity insured by the manufacturer on the day of sampling until the end of the incubation period.

At the sample location aseptically remove a sterile gelatine filter from its packaging and mount it on the air sampler. Collect air with a volume flow of 30 liter/min with the gelatine filter exposed horizontally. The volume of air sampled with each gelatine filter shall not exceed 300 liters.

After collection aseptically remove gelatine filter from the air sampler and pack filter in a sterile transport container.

G.1.3. Transport and storage

Transport samples to the laboratory and store at (4 – 8)°C and process within 24 hours.

G.1.4. Incubation

Aseptically remove the upper part of the filter holder from the gelatine filter. Press filter slightly on the surface of a R2A plate for a few seconds until the filter sticks on the agar and discard the lower part of the filter holder.

Plates shall be incubated inverted at (32±1)°C.

G.1.5. Counting

Examine the sample plates at 24 and 48 hours. If colonies visible by eyes are observed, count and record data. Examine and record final colony counts at 72 hours. Do not remove the Petri plate covers until the final 72 hour count is made.

G.1.6. Controls

For each ten or fewer samples collected, also collect a 'field negative' control, at least 3 per day obtained according to the procedure described above but without collecting air with the air sampler.

In the lab, create at least two 'lab negative controls' obtained according to the procedure described above but without collecting air with the air sampler.

Analyse the controls in the same way as the samples described above.

G.1.7. Equipment, reagents and consumable materials

- Portable active air sampler with a rechargeable battery
- Sterile ready-to-connect gelatine membrane filter units with a filtration area of 38.5 cm²
- Sterile transport bags
- R2A plates (90 mm)
- Refrigerator (4 – 8) °C
- Laminar flow hood
- Incubator (32 ± 1)°C
- Sterile gloves

Applicability matrix

Identifier	Requirement	Applicable (A/M/N)	Modified requirement

Bibliography

ISO 14698 part 1	Cleanrooms and associated controlled environments - Biocontamination control - Part 1: General principles and methods
ISO 14698 part 2	Cleanrooms and associated controlled environments - Biocontamination control - Part 2: Evaluation and interpretation of biocontamination data
IEST-RP-CC023.2	Microorganisms in Cleanrooms
NASA NPG 5350	Standard procedures for the microbiological examination of space hardware